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(54) Title: THERAPIES THAT IMPROVE GRAFT SURVIVAL, USING ANTIBODIES AGAINST A B7 ANTIGEN

(57) Abstract: The invention provides methods for downmodulating the immune response in a subject undergoing transplantation comprising administering to the subject at least one antibody that recognizes a B7 antigen according to specific treatment protocols.

THERAPIES THAT IMPROVE GRAFT SURVIVAL, USING ANTIBODIES AGAINST A B7 ANTIGEN

Related Application

The present application claims priority to U.S. Provisional Patent Application Serial No. 60/189,164, filed March 14, 2000, entitled "Antibody Therapies that Improve Graft Survival", the entire contents of which are expressly incorporated by reference.

Background of the Invention

In order for T cells to respond to foreign proteins, two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144, 3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow et al. 1996. *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. 1988 *J. Immunol.* 140, 3324-3330; Linsley, P.S., et al. 1991 *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al., 1991 *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; Young, J.W., et al. 1992 *J. Clin. Invest.* 90, 229-237; Koulova, L., et al. 1991 *J. Exp. Med.* 173, 759-762; Reiser, H., et al. 1992 *Proc. Natl. Acad. Sci. USA.* 89, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144, 4579-4586; LaSalle, J.M., et al., 1991 *J. Immunol.* 147, 774-80; Dustin, M.I., et al., 1989 *J. Exp. Med.* 169, 503; Armitage, R.J., et al. 1992 *Nature* 357, 80-82; Liu, Y., et al. 1992 *J. Exp. Med.* 175, 437-445).

The CD80 (B7-1) and CD86 (B7) proteins, expressed on APCs, are critical costimulatory molecules (Freeman et al. 1991. *J. Exp. Med.* 174:625; Freeman et al. 1989 *J. Immunol.* 143:2714; Azuma et al. 1993 *Nature* 366:76; Freeman et al. 1993. *Science* 262:909). B7 appears to play a predominant role during primary immune responses, while B7-1, which is upregulated later in the course of an immune response,

may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone. 1995. *Immunity.* 2:555).

One ligand to which B7-1 and B7-2 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal induces T cells to proliferate and secrete IL-2 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:6575-6579; June, C.H. *et al.* (1990) *Immunol. Today* 11:211-6; Harding, F. A. *et al.* (1992) *Nature* 356:607-609). A second ligand, termed CTLA4 (CD152) is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J. F. *et al.* (1987) *Nature* 328:267-270). In contrast to CD28, CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse *et al.* (1995) *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate T cell responses (Allison and Krummel (1995) *Science* 270:932). The B7 molecules have a higher affinity for CTLA4 than for CD28 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 174:561-569) and B7-1 and B7-2 have been found to bind to distinct regions of the CTLA4 molecule and have different kinetics of binding to CTLA4 (Linsley *et al.* (1994) *Immunity* 1:793).

A new molecule related to CD28 and CTLA4, ICOS, has been identified (Hutloff *et al.* (1999) *Nature* 397:263; WO 98/38216; Tamatani, T. *et al.* (2000) *Int. Immunol.* 12:51-55), as has its ligand, GL50 (also called by the names ICOSL, B7h, LICOS, and B7RP-1) which is a new B7 family member (Ling, V. *et al.* (2000) *J. Immunol.* 164:1653-7; Swallow, M. M. *et al.* (1999) *Immunity* 11:423-432; Aicher, A. *et al.* (2000) *J. Immunol.* 164:4689-96; Mages, H. W. *et al.* (2000) *Eur. J. Immunol.* 30:1040-7; Brodie, D. *et al.* (2000) *Curr. Biol.* 10:333-6; Yoshinaga, S. K. *et al.* (1999) *Nature* 402:827-32). An additional B7 family member, B7-H1, has also been identified (Dong, H. *et al.* (1999) *Nat. Med.* 5:1365-1369). B7-H1, also known as PD-L1, interacts with the immunoinhibitory receptor PD-1 (Freeman, G. J. *et al.* (2000) *J. Exp. Med.* 192:1027-34).

If T cells are only stimulated through the T cell receptor, without receiving an additional costimulatory signal, they become nonresponsive, anergic, or die, resulting in downmodulation of the immune response. The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and humans systems (Harding, F.A., et al. (1992) *Nature.* 356, 607-609; Lenschow, D.J., et al. (1992) *Science.* 257, 789-792; Turka, L.A., et al. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 11102-11105; Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci USA* 90, 6586-6590; Boussiotis, V., et al. (1993) *J. Exp. Med.* 178, 1753-1763). Conversely, expression of B7 by B7 negative murine tumor cells induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L., et al. (1992) *Cell* 71, 1093-1102; Townsend, S.E. and Allison, J.P. (1993) *Science* 259, 368-370; Baskar, S., et al. (1993) *Proc. Natl. Acad. Sci.* 90, 5687-5690.). Therefore, manipulation of the costimulatory pathways offers great potential to stimulate or suppress immune responses in humans.

Summary of the Invention

In one aspect, the invention pertains to a method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject at least one antibody that recognizes a B7 antigen immediately prior to surgery and postoperatively administering to the subject at least one antibody that recognizes a B7 antigen immediately following surgery.

In one embodiment, the method further comprises preoperatively administering at least one antibody that recognizes a B7 antigen at least about four days prior to surgery.

In one embodiment two antibodies that recognize at least two B7 antigens are administered to the subject.

In one embodiment, at least one antibody is a humanized antibody.

In one embodiment, a higher dose of at least one antibody is administered prior to surgery than after surgery.

In one embodiment, the method comprises postoperatively administering at least one antibody that recognizes a B7 antigen at weekly intervals for at least about 3 months.

In one aspect, the invention pertains to a method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject at least one antibody that recognizes a B7 antigen and a postoperatively administering to the subject at least one antibody that recognizes a B7 antigen in combination with an immunosuppressive drug.

In another aspect, the invention pertains to a method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject a combination of antibodies that recognize at least two B7 antigens and postoperatively administering to the subject a combination of antibodies that recognize at least two B7 antigens in combination with an immunosuppressive drug.

In one embodiment, the immunosuppressive drug is a rapamycin compound.

Brief Description of the Figures

Figure 1 shows mean plasma trough levels of humanized monoclonal antibodies (h3DI and hlFI) expressed in ug/L. Antibody administration schedule included weekly administration of both mAb at 5 mg/kg until poday 56. After cessation of antibody administration, levels were detectable until approximately post operative day (poday) 100, which shows that the primates did not develop antibodies against the humanized monoclonals.

Figure 2 shows average CsA dose in mg/kg of group II and III animals. CsA dose was adjusted to meet target trough levels (see also Figure 3).

Figure 3 shows average 24 hour cyclosporine trough levels of group II and III. The target 24 hour trough level for the first 14 postoperative days was 200 to 300 ng/ml and 150 to 250 ng/ml from po day 15 to poday 56. There were no significant differences in CsA trough levels during the period of administration by ANOVA with repeated measures analysis.

Figure 4 shows average change in animal weight calculated as a percentage change from the animal's weight at the time of transplantation. All animals experienced a substantial weight loss during the first 3 week postoperative weeks but then nearly regained their lost weight or at least ceased to continue showing weight loss.

Figure 5 shows core body temperatures of individual monkeys in the 4 treatment groups measured by telemetry. Each animal in group I developed a febrile episode between poday 4 and poday 8, while combined therapy with mAb and cyclosporine or prednisone prevented temperature increases above 39 C in all monkeys in group II and IV. In group III (cyclosporine monotherapy) 2 of 4 animals had febrile episodes around poday 6.

Figure 6 shows clinical and histopathological course of individual monkeys in the 4 treatment groups. The horizontal bars represent the length of survival. The final histological diagnosis is shown at the end of the survival bar. Biopsy results at poday 7, 28 and 70 are depicted as open blocks unless the results showed no evidence of rejection. No biopsies were obtained from the animals in group I. Clinical rejection episodes are marked as solid blocks along the postoperative course and graded as being mild, moderate or severe (mild clinical rejection is defined as a febrile episode without significant changes in serum creatinine levels; moderate clinical rejection is defined as moderate increases in serum creatinine with or without febrile episodes; severe clinical rejection is defined as significant increases in serum creatinine with fever and oliguria).

Detailed Description of the Invention

The instant invention provides improved methods of downmodulating immune responses by administering at least one agent that blocks a B7 activity to a subject undergoing transplantation according to an optimized dosage regimen. In a preferred embodiment, the methods employ a combination therapy, wherein at least two agents that block an activity of at least two different B7 molecules are administered to a subject undergoing transplantation according to an optimal dosage regimen. In one embodiment, the methods further comprise administering an immunosuppressive drug, e.g, a rapamycin or steroid compound.

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

Definitions

As used herein, the term "combination therapy" includes a combination of at least two agents which block the activity of at least two B7 molecules. For example, an antibody that binds to B7-1 and an antibody that binds to B7-2 can be used in a combination therapy. The term "combination therapy" also includes at least one agent that blocks the activity of a B7 molecule used in combination with an immunosuppressive drug.

As used herein, the term " immune response" includes T and/or B cell responses, i.e., cellular and/or humoral immune responses. In one embodiment, the claimed methods can be used to reduce T helper cell responses. In another embodiment, the claimed methods can be used to reduce cytotoxic T cell responses. The claimed methods can be used to reduce both primary and secondary immune responses. The immune response of a subject can be determined by, for example, assaying antibody production, immune cell proliferation, the release of cytokines, the expression of cell surface markers, cytotoxicity, etc.

As used herein, the term "costimulate" with reference to activated immune cells includes the ability of a costimulatory molecule to provide a second, non- activating receptor mediated signal (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, e.g., in a T cell that has received a T cell-receptor-mediated signal. As used herein the term "costimulatory molecule" includes molecules which are present on antigen presenting cells (e.g., B7-1, B7-2, B7RP-1 (Yoshinaga et al. 1999. Nature 402:827), B7h (Swallow et al. 1999. Immunity. 11:423) and/or related molecules (e.g., homologs)) that bind to costimulatory receptors (e.g., CD28, CTLA4, ICOS (Hutloff et al. 1999. Nature 397:263), B7h ligand (Swallow et al. 1999. Immunity. 11:423) and/or related molecules) on T cells. These molecules are also collectively referred to herein as "B7 molecules."

As used herein, the language "B7" or "B7 molecule" includes naturally occurring B7-1 molecules, B7-2 molecules, B7RP-1 molecules (Yoshinaga et al. 1999. Nature 402:827), B7h molecules (Swallow et al. 1999. Immunity. 11:423), structurally related molecules, fragments of such molecules, and/or functional equivalents thereof. The term "equivalent" is intended to include amino acid sequences encoding functionally equivalent costimulatory molecules having an activity of a B7 molecule, e.g., the ability to bind to the natural ligand(s) of B7 on immune cells, such as CTLA4, ICOS, PD-1, and/or CD28 on T cells, and the ability to modulate immune cell costimulation.

As used here, the term "agent that blocks a B7 activity" includes those agents that interfere with the ability of a B7 molecule to bind its natural ligand and/ or that interfere with the ability of a B7 molecule to costimulate T cells, e.g., as measured by cytokine production and/or proliferation. Exemplary agents include blocking antibodies, peptides that block the ability of B7 to bind to its natural ligand but which fail to transmit a costimulatory signal to a T cell, peptidomimetics, small molecules, and the like.

The term "antibody", as used herein, includes immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The phrase "complementary determining region" (CDR) includes the region of an antibody molecule which comprises the antigen binding site.

The antibody may be an IgG such as IgG1, IgG2, IgG3 or IgG4; or IgM, IgA, IgE or IgD isotype. The constant domain of the antibody heavy chain may be selected depending upon the effector function desired. The light chain constant domain may be a kappa or lambda constant domain.

The term "antibody" as used herein also includes an "antigen-binding portion" of an antibody (or simply "antibody portion"). The term "antigen-binding portion", as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, the extracellular domain of a B7 molecule). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883) . Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123).

Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof, e.g. humanized, chimeric, etc. Preferably, antibodies of the invention bind specifically or substantially specifically to B7 molecules. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition, typically displays a single binding affinity for a particular antigen with which it immunoreacts.

The term "humanized antibody", as used herein, includes antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The

term "humanized antibody", as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

An "isolated antibody", as used herein, includes an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds B7 is substantially free of antibodies that specifically bind antigens other than B7). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

As used herein, the term "extracellular domain of a B7 molecule" includes a portion of a B7 molecule which, in the cell-associated form of a B7 molecule, is extracellular. A B7 extracellular domain includes the portion of a B7 molecule which mediates binding to a costimulatory receptor, e.g., CD28, ICOS, PD-1 and/or CTLA4. For example, the human B7-1 extracellular domain comprises from about amino acid 1 to about amino acid 208 and the human B7 extracellular domain comprises from about amino acid 24 to about amino acid 245. See, for example, B7-2 (Freeman et al. 1993 *Science*. 262:909; GenBank Accession numbers P42081 or A48754; or United States Patent 5,942,607); B7-1 (Freeman et al. *J. Exp. Med.* 1991. 174:625; GenBank Accession numbers P33681 or A45803; or United States Patent 5,858,776).

The language "a desired binding specificity for a B7 epitope", as well as the more general language "an antigen binding site which specifically binds (immunoreacts with)", refers to the ability of individual antibodies to specifically immunoreact with a peptide having a B7 costimulatory activity. That is, it refers to a non-random binding reaction between an antibody molecule and an antigenic determinant of B7. Illustrative of a specific antibody-antigen complex is that between antibody 2D10 and mouse B7-2 (J Immunol 1994 152: 2105-14). The desired binding specificity is typically determined from the reference point of the ability of the antibody to differentially bind a B7 antigen and an unrelated antigen, and therefore distinguish between two different antigens - particularly where the two antigens have unique epitopes. In other embodiments, the desired binding affinity refers to the ability of the antibody to discriminate in binding between different isoforms of B7 antigens or between different B7 antigens. An antibody which binds specifically to a B7 epitope is referred to as a "specific antibody".

Preferably, the anti-B7 antibodies of the invention bind to naturally occurring forms of B7, but are substantially unreactive, e.g., have background binding to unrelated, non-B7 molecules. Antibodies specific for a B7 molecule from one source, e.g., human B7-1 may or may not be reactive with B7-1 molecules from different species. In addition, antibodies specific for naturally occurring B7 molecules may or may not bind to mutant forms of such molecules. In one embodiment, mutations in the amino acid sequence of a naturally occurring B7 molecule result in modulation of the binding (e.g., either increased or decreased binding) of the antibody to the B7 molecule. Antibodies to B7 molecules can be readily screened for their ability to meet this criteria. Assays to determine affinity and specificity of binding are known in the art, including competitive and non-competitive assays. Assays of interest include ELISA, RIA, flow cytometry, etc. Binding assays may use purified or semi-purified B7 protein, or alternatively may use cells that express B7, e.g. cells transfected with an expression construct for B7. As an example of a binding assay, purified B7 protein is bound to an insoluble support, e.g. microtiter plate, magnetic beads, etc. The candidate antibody and soluble, labeled CTLA4 or CD28 are added to the cells, and the unbound components are then washed off. The ability of the antibody to compete with CTLA4 or CD28 for B7 binding is determined by quantitation of bound, labeled CTLA4 or CD28. An isolated antibody that specifically binds human B7 may, however, have cross-reactivity to other antigens, such as B7 molecules from other species.

"Antibody combining site", as used herein, refers to that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) antigen. The term "immunoreact" or "reactive with" in its various forms is used herein to refer to binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

The term "antigenic determinant", as used herein, refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site. The term is also used interchangeably with "epitope".

II. B7 Molecules and Agents that Block B7 Activity

The B7 antigens are a family of costimulatory molecules found on the surface of B lymphocytes, professional antigen presenting cells (e.g., monocytes, dendritic cells, Langerhan cells) and cells which present antigen to immune cells (e.g., keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes). These costimulatory molecules bind either CTLA4, CD28, PD-1 and/or ICOS on the surface of T cells or other known or as yet undefined receptors on immune cells. The members of this family of costimulatory molecules are capable of providing costimulation to activated T cells to thereby induce T cell proliferation and/or cytokinc secretion.

Agents that block an activity of a B7 molecule can be derived using B7 nucleic acid or amino acid sequences. For example, nucleotide sequences of costimulatory molecules are known in the art and can be found in the literature or on a database such as GenBank. See, for example, B7-2 (Freeman et al. 1993 *Science*. 262:909 or GenBank Accession numbers P42081 or A48754); B7-1 (Freeman et al. *J. Exp. Med.* 1991. 174:625 or GenBank Accession numbers P33681 or A45803; CTLA4 (See e.g., Ginsberg et al. 1985. *Science*. 228:1401; or GenBank Accession numbers P16410 or 291929); and CD28 (Aruffo and Seed. *Proc Natl. Acad. Sci.* 84:8573 or GenBank Accession number 180091), ICOS (Hutloff et al. 1999. *Nature*. 397:263; WO 98/38216), PD-1 (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704) and related sequences. Purification techniques for B7 molecules have been established, and, additionally, B7 genes (cDNA) have been cloned from a number of species, including human and mouse (see, for example, Freeman, G.J. et al. (1993) *Science* 262:909-911; Azuma, M. et al. (1993) *Nature* 366:76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178:2185-2192).

Such nucleic acid molecules and polypeptides can be used to derive agents that block a B7 activity. For example, nucleic acid molecules that are antisense to a B7 molecule can be used to inhibit expression of B7 and thereby block a B7 activity.

Non-naturally occurring forms, e.g., mutant forms of costimulatory molecules can also be used to derive agents of the invention. For example, DNA sequences capable of hybridizing to DNA encoding a B7 molecule, under conditions that avoid hybridization to non-costimulatory molecule genes, (e.g., under conditions equivalent to

65°C in 5 X SSC (1 X SSC = 150 mM NaCl/ 0.15 M Na citrate)) can be used to make antiB7 antibodies. Alternatively, DNA sequences which retain sequence identity over regions of the nucleic acid molecule which encode protein domains which are important in costimulatory molecule function, e.g., binding to other costimultory molecules, can be used to produce costimulatory proteins which can be used as immunogens. Preferably, nonnaturally occurring costimulatory molecules have significant (e.g., greater than 70%, preferably greater than 80%, and more preferably greater than 90-95%) amino acid identity with a naturally occurring amino acid sequence of a costimulatory molecule extracellular domain.

In making non-naturally occurring variants of costimulatory molecules, amino acid residues which are likely to be important in the binding of a costimulatory molecule to its counter receptor, amino acid sequences comprising the extracellular domains of costimulatory molecules of different species, e.g., mouse and human, can be aligned and conserved (e.g., identical) residues noted. This can be done, for example, using any standard alignment program, such as MegAlign (DNA STAR). Such conserved or identical residues are likely to be necessary for proper binding of costimulatory molecules to their receptors and are, thus, not likely to be amenable to alteration.

For example, the regions of a B7 molecule which are important in mediating the functional interaction with CD28 and CTLA4 have been identified by mutation. Two hydrophobic residues in the V-like domain of B7-1, including the Y87 residue, which is conserved in all B7-1 and B7-2 molecules cloned from various species, were found to be critical (Fargeas et al. 1995. *J. Exp. Med.* 182:667). Using these, or similar, techniques amino acid residues of the extracellular domains of costimulatory molecules which are critical and, therefore, not amenable to alteration can be determined.

Using B7 cDNA molecules, peptides derived from the B7 sequence can be produced using standard techniques. Host cells transfected to express peptides can be any procaryotic or eucaryotic cell. For example, a peptide can be expressed in bacterial cells such as *E. coli,* insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) and NS0 cells. Other suitable host cells and expression vectors may be found in Goeddel, (1990) *supra* or are known to those skilled in the art. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1

(Baldari. et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM8 (Seed, B., (1987) *Nature* 329:840) for transient amplification/expression in mammalian cells, while CHO (dhfr- Chinese Hamster Ovary) cells are used with vectors such as pMT2PC (Kaufman et al. (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. A preferred cell line for production of recombinant protein is the NS0 myeloma cell line available from the ECACC (catalog #85110503) and described in Galfre, G. and Milstein, C. ((1981) *Methods in Enzymology* 73(13):3-46; and *Preparation of Monoclonal Antibodies: Strategies and Procedures*, Academic Press, N.Y., N.Y.). Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, or electroporation. Suitable methods for transforming host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks. When used in mammalian cells, the expression vector's control functions are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and most frequently, Simian Virus 40.

Polypeptides expressed in mammalian cells or otherwise can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, electrophoresis, affinity chromatography, etc.) and ultimately, crystallization (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)).

The present invention also pertains to variants of the B7 polypeptides which function as B7 antagonists. Variants of the B7 polypeptides can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a B7 polypeptide. An agonist of the B7 polypeptide can retain substantially the same, or a subset, of the biological

activities of the naturally occurring form of a B7 polypeptide. An antagonist of a B7 polypeptide can inhibit one or more of the activities of the naturally occurring form of the B7 polypeptide by, for example, competitively modulating a cellular activity of a B7 polypeptide. Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the B7 polypeptide.

In one embodiment, variants of a B7 polypeptide which function as either B7 antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a B7 (or B7 ligand) polypeptide for B7 antagonist activity. In one embodiment, a variegated library of B7variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of B7 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential B7 or B7 ligand sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of B7 or B7 ligand sequences therein. There are a variety of methods which can be used to produce libraries of potential B7 or B7 ligand variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential B7 or B7 ligand sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a B7 or B7 ligand coding sequence can be used to generate a variegated population of B7 or B7 ligand fragments for screening and subsequent selection of variants of a B7 or B7 ligand polypeptide. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded

PCR fragment of a B7 or B7 ligand coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the B7 or B7 ligand.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of B7 or B7 ligand proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify B7 or B7 ligand variants (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated B7 or B7 ligand library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes B7 or B7 ligand. The transfected cells are then cultured such that B7 or B7 ligand and a particular mutant B7 or B7 ligand are secreted and the effect of expression of the mutant on B7 or B7 ligand activity can be detected, *e.g.*, by any of a number of functional assays. DNA can then be recovered from the cells which score for inhibition of B7or B7 ligand activity, and the individual clones further characterized.

In addition to B7 or B7 ligand polypeptides consisting only of naturally-occurring amino acids, B7 or B7 ligand peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) TINS p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as human B7 or B7 ligand, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods known in the art and further described in the following references: Spatola, A. F. in "*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*" Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH2NH-, CH2CH2-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH2-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans. I.* 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH2-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH2-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH2-); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH2-); and Hruby, V. J. (1982) *Life Sci.* (1982) 31:189-199 (-CH2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a

broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Systematic substitution of one or more amino acids of a B7 or B7 ligands amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a B7 or B7 ligand amino acid sequence or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of B7 or B7 ligand polypeptides identified herein will enable those of skill in the art to produce polypeptides corresponding to B7 or B7 ligand peptide sequences and sequence variants thereof. Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a B7 or B7 ligand peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.*

57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Peptides can be produced, typically by direct chemical synthesis, and used *e.g.*, as agonists or antagonists of a B7/ B7 ligand interaction. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient. Peptidomimetics can be made as described, e.g., in WO 98/56401.

An isolated B7 or B7 ligand protein, or a portion or fragment thereof (or a nucleic acid molecule encoding such a polypeptide), can be used as an immunogen can also be used to make an antibody that blocks a B7 activity. In one embodiment, antibodies for use in the instant methods bind to at least one B7 molecule. In yet another embodiment, an antibody of the invention binds to only one B7 molecule (e.g., to B7-1 and not to B7-2). Such antibodies are known in the art. For example, The 2D10 hybridoma, producing the 2D10 antibody, has been described (Journal of Immunology. 1994. 152:2105). In addition, for use in combination with an anti-B7-2 antibody, several anti-B7-1 antibodies are known or are readily available (see, e.g., United States Patent 5,869,050; Powers G.D., et al. (1994) *Cell. Immunol.* 153, 298-311; Freedman, A.S. et al. (1987) *J. Immunol.* 137:3260-3267; Freeman, G.J. et al. (1989) *J. Immunol.* 143:2714-2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174:625-631; Freeman, G.J. et al. (1993) *Science* 262:909-911; WO 96/40915). Such antibodies are also commercially

available, e.g., from R&D Systems (Minneapolis, MN) and from Research Diagnostics (Flanders, NJ)

Moreover, it will be appreciated by those skilled in the art that it is within their skill to generate additional agents and screen for their activity by following standard techniques. For instance, B7 molecules from a variety of species, whether in soluble form or membrane bound, can be used to induce the formation of anti-B7 antibodies. Such antibodies may either be polyclonal or monoclonal, or antigen binding fragments of such antibodies. Of particular significance for use in therapeutic applications are antibodies that inhibit binding of B7 with its natural ligand(s) on the surface of immune cells, thereby inhibiting costimulation of the immune cell through the B7-ligand interaction. Preferred anti-B7 antibodies are those capable of inhibiting or downregulating T cell mediated immune responses by binding B7 on the surface of B lymphocytes and preventing interaction of B7 with CTLA4 and/or CD28. Preferably, the combination of antibodies chosen for use in the invention results in increased inhibition of costimulation of an immune cell, such as a T cell, through the B7-ligand interaction, relative to either antibody alone.

A. The Immunogen. The term "immunogen" is used herein to describe a composition containing a peptide derived from the amino acid sequence of a B7 molecule as an active ingredient used for the preparation of antibodies against a B7 molecule. When a peptide derived from the amino acid sequence of a B7 molecule is used to induce antibodies it is to be understood that the peptide can be used alone, or linked to a carrier as a conjugate, or as a peptide polymer.

Peptides derived from the amino acid sequence of a B7 molecule expressed in mammalian cells or otherwise can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, electrophoresis, affinity chromatography, etc.) and ultimately, crystallization (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)).

To generate suitable anti- B7 molecule antibodies, the immunogen should contain an effective, immunogenic amount of a peptide having a B7 molecule activity, typically as a conjugate linked to a carrier. The effective amount of peptide per unit

dose depends, among other things, on the species of animal inoculated, the body weight of the animal and the chosen immunization regimen as is well known in the art. The immunogen preparation will typically contain peptide concentrations of about 10 micrograms to about 500 milligrams per immunization dose, preferably about 50 micrograms to about 50 milligrams per dose. An immunization preparation can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

Those skilled in the art will appreciate that, instead of using naturally occurring forms of a B7 molecule for immunization, synthetic peptides can alternatively be employed towards which antibodies can be raised for use this invention. Both soluble and membrane bound costimulatory molecule or peptide fragments are suitable for use as an immunogen and can also be isolated by immunoaffinity purification as well. A purified form of a B7 molecule protein, such as may be isolated as described above or as known in the art, can itself be directly used as an immunogen, or alternatively, can be linked to a suitable carrier protein by conventional techniques, including by chemical coupling means as well as by genetic engineering using a cloned gene of the a costimulatory molecule.

The peptide or protein chosen for immunization can be modified to increase its immunogenicity. For example, techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. Any peptide chosen for immunization can also be synthesized. In certain embodiments, such peptides can be synthesized as branched polypeptides, to enhance immune responses, as is known in the art (see, e.g., Peptides. Edited by Bernd Gutte Academic Press 1995. pp. 456-493).

The purified B7 molecule protein can also be covalently or noncovalently modified with non-proteinaceous materials such as lipids or carbohydrates to enhance immunogenecity or solubility. Alternatively, a purified B7 molecule protein can be coupled with or incorporated into a viral particle, a replicating virus, or other microorganism in order to enhance immunogenicity. The B7 molecule protein may be,

for example, chemically attached to the viral particle or microorganism or an immunogenic portion thereof.

In an illustrative embodiment, a purified B7 molecule protein, or a peptide fragment having a B7 molecule activity (e.g., produced by limited proteolysis or recombinant DNA techniques) is conjugated to a carrier which is immunogenic in animals. Preferred carriers include proteins such as albumin, serum proteins (e.g., globulins and lipoproteins), and polyamino acids. Examples of useful proteins include bovine serum albumin, rabbit serum albumin, thyroglobulin, keyhole limpet hemocyanin, egg ovalbumin and bovine gamma-globulins. Synthetic polyamino acids such as polylysine or polyarginine are also useful carriers. With respect to the covalent attachment of a B7 molecule protein or peptide fragments to a suitable immunogenic carrier, there are a number of chemical cross-linking agents that are known to those skilled in the art. Preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link proteins in a stepwise manner. A wide variety of heterobifunctional cross-linkers are known in the art, including succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidyloxycarbonyl-a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP).

In may also be desirable to simply immunize with whole cells which express a costimulatory molecule protein on their surface. Various cell lines can be used as immunogens to generate monoclonal antibodies to a B7 molecule antigen, including, but not limited to activated B cells. For example, splenic B cells can be obtained from a subject and activated with anti-immunoglobulin. Alternatively, a B cell line can be used, provided that a costimulatory molecule is expressed on the cell surface, such as the Raji cell line (B cell Burkett's lymphoma, see e.g., Freeman, G.J. et al. (1993) *Science* 262:909-911) or the JY B lymphoblastoid cell line (see e.g., Azuma, M. et al. (1993) *Nature* 366:76-79). Whole cells that can be used as immunogens to produce

costimulatory molecule specific antibodies also include recombinant transfectants. For example, COS and CHO cells can be reconstituted by transfection with a costimulatory molecule cDNA, such as described by Knudson et al. (1993, *PNAS* 90:4003-4007); Travernor et al. (1993, *Immunogenitics* 37:474-477); Dougherty et al. (1991, *J Exp Med* 174:1-5); and Aruffo et al. (1990, *Cell* 61:1303-1313), to produce intact costimulatory molecule on the cell surface. These transfectant cells can then be used as immunogen to produce anti-costimulatory molecule antibodies of preselected specificity. Other examples of transfectant cells are known, particularly eukaryotic cells able to glycosylate the costimulatory molecule protein, but any procedure that works to express transfected costimulatory molecule genes on the cell surface could be used to produce the whole cell immunogen.

B. Polyclonal Anti-Costimulatory Molecule Antibodies.

Polyclonal anti-B7 antibodies can generally be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of a B7 molecule immunogen, such as the extracellular domain of a B7 molecule protein, and an adjuvant. For example, as described above, it may be useful to conjugate a B7 molecule (including fragments containing particular eptitope(s) of interest) to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin.

The route and schedule of the host animal or antibody-producing cells cultured therefrom can generally make use of established and conventional techniques for antibody stimulation and production. In an illustrative embodiment, animals are typically immunized against the immunogenic B7 molecule conjugates or derivatives by combining about 1μg to 1mg of conjugate with Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for anti-costimulatory molecule titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same costimulatory molecule protein, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in

recombinant cell culture as protein fusions. Also, aggregating agents such as alum can be used to enhance the immune response.

Such mammal-produced populations of antibody molecules are referred to as "polyclonal" because the population comprises antibodies with differing immunospecificities and affinities for a costimulatory molecule. The antibody molecules are then collected from the mammal and isolated by well known techniques such as, for example, by using DEAE Sephadex to obtain the IgG fraction. To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid phase-affixed immunogen. The antibody is contacted with the solid phase-affixed immunogen for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. The bound antibodies are separated from the complex by standard techniques.

C. Monoclonal Anti-Costimulatory Molecule Antibodies. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of a B7 molecule. A monoclonal antibody composition thus typically displays a single binding affinity for a particular B7 molecule protein with which it immunoreacts. Preferably, the monoclonal antibody used in the subject method is further characterized as immunoreacting with a B7 molecule derived from humans.

Monoclonal antibodies useful in the compositions and methods of the invention are directed to an epitope of a B7 molecule antigen, such that complex formation between the antibody and the B7 molecule antigen inhibits interaction of the B7 molecule with its natural ligand(s) on the surface of immune cells, thereby inhibiting costimulation of a T cell through the B7 molecule-ligand interaction. A monoclonal antibody to an epitope of a B7 molecule can be prepared by using a technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497), and the more recent human B cell

hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96), and trioma techniques. Other methods which can effectively yield monoclonal antibodies useful in the present invention include phage display techniques (Marks et al. (1992) *J Biol Chem* 16007-16010).

In one embodiment, the antibody preparation applied in the subject method is a monoclonal antibody produced by a hybridoma cell line. Hybridoma fusion techniques were first introduced by Kohler and Milstein (Kohler et al. *Nature* (1975) 256:495-97; Brown et al. (1981) *J. Immunol* 127:539-46; Brown et al. (1980) *J Biol Chem* 255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75). Thus, the monoclonal antibody compositions of the present invention can be produced by the following method, which comprises the steps of:

(a) Immunizing an animal with a B7 molecule. The immunization is typically accomplished by administering a B7 molecule immunogen to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period sufficient for the mammal to produce cells secreting antibody molecules that immunoreact with the B7 molecule immunogen. Such immunoreaction is detected by screening the antibody molecules so produced for immunoreactivity with a preparation of the immunogen protein. Optionally, it may be desired to screen the antibody molecules with a preparation of the protein in the form in which it is to be detected by the antibody molecules in an assay, e.g., a membrane-associated form of a B7 molecule. These screening methods are well known to those of skill in the art.

(b) A suspension of antibody-producing cells removed from each immunized mammal secreting the desired antibody is then prepared. After a sufficient time, the mouse is sacrificed and somatic antibody-producing lymphocytes are obtained. Antibody-producing cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiologically tolerable medium using methods well known in the art. Mouse lymphocytes give a higher percentage of stable fusions with the mouse

myelomas described below. Rat, rabbit and frog somatic cells can also be used. The spleen cell chromosomes encoding desired immunoglobulins are immortalized by fusing the spleen cells with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of antibody of the desired specificity, e.g., by immunoassay techniques using the antigen that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions and the monoclonal antibody produced can be isolated. Various conventional methods exist for isolation and purification of the monoclonal antibodies so as to free them from other proteins and other contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see, e.g., Zola et al. in Monoclonal Hybridoma Antibodies: Techniques And Applications, Hurell (ed.) pp. 51-52 (CRC Press 1982)). Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art.

Generally, the individual cell line may be propagated *in vitro*, for example in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation. Alternatively, the yield of monoclonal antibody can be enhanced by injecting a sample of the hybridoma into a histocompatible animal of the type used to provide the somatic and myeloma cells for the original fusion. Tumors secreting the specific monoclonal antibody produced by the fused cell hybrid develop in the injected animal. The body fluids of the animal, such as ascites fluid or serum, provide monoclonal antibodies in high concentrations. When human hybridomas or EBV-

hybridomas are used, it is necessary to avoid rejection of the xenograft injected into animals such as mice. Immunodeficient or nude mice may be used or the hybridoma may be passaged first into irradiated nude mice as a solid subcutaneous tumor, cultured *in vitro* and then injected intraperitoneally into pristane primed, irradiated nude mice which develop ascites tumors secreting large amounts of specific human monoclonal antibodies.

Media and animals useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al. (1959) *Virol* 8:396) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

D. Humanized or Chimeric Anti- B7 Molecule Antibodies. When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Such antibodies are the equivalents of the monoclonal and polyclonal antibodies described above, but may be less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) reactive with a costimulatory molecule can be produced, for example, by techniques recently developed for the production of chimeric antibodies. Methods of humanizing antibodies are known in the art. The humanized antibody may be the product of an animal having transgenic human immunoglobulin constant region genes (see for example International Patent Applications WO 90/10077 and WO 90/04036). Alternatively, the antibody of interest may be engineered by recombinant DNA techniques to substitute the CH1, CH2, CH3, hinge domains, and/or the framework domain with the corresponding human sequence (see WO 92/02190).

The use of Ig cDNA for construction of chimeric immunoglobulin genes is known in the art (Liu et al. (1987) P.N.A.S. 84:3439 and (1987) J. Immunol. 139:3521). mRNA is isolated from a hybridoma or other cell producing the antibody and used to produce cDNA. The cDNA of interest may be amplified by the polymerase chain reaction using specific primers (U.S. Pat. Nos. 4,683,195 and 4,683,202). Alternatively, a library is made and screened to isolate the sequence of interest. The DNA sequence encoding the variable region of the antibody is then fused to human constant region sequences. The sequences of human constant regions genes may be found in Kabat et al. (1991) Sequences of Proteins of Immunological Interest, N.I.H. publication no. 91-3242. Human C region genes are readily available from known clones. The choice of isotype will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity. Preferred isotypes are IgG1, IgG3 and IgG4. Either of the human light chain constant regions, kappa or lambda, may be used. The chimeric, humanized antibody is then expressed by conventional methods.

Additionally, recombinant anti-B7 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060. In addition, humanized antibodies can be made according to standard

protocols such as those disclosed in US patents 5,777,085; 5,530,101; 5,693,762; 5,693,761; 5,882,644; 5834597; 5932448; or 5,565,332.

Fully human anti-B7 antibodies may also be made by immunizing animals (e.g., mice) transgenic for human immunoglobulin genes using the methods of Lonberg and Huszar (1995) *Int. Rev. Immunol.* 13:65-93; Lonberg *et al.* US Patent Nos. 5,877,397, 5,874,299, 5,814,318, 5,789,650, 5,770,429, 5,661,016, 5,633,425, 5,625,126, 5,569,825, and 5,545,806; and Kucherlapati *et al.* US Patent Nos. 6,162,963, 6,150,584, 6,114,598, and 6,075,181.

For example, an antibody may be humanized by grafting the desired CDRs onto a human framework, e.g., according to EP-A-0239400. A DNA sequence encoding the desired reshaped antibody can therefore be made beginning with the human DNA whose CDRs it is wished to reshape. The rodent variable domain amino acid sequence containing the desired CDRs is compared to that of the chosen human antibody variable domain sequence. The residues in the human variable domain are marked that need to be changed to the corresponding residue in the rodent to make the human variable region incorporate the rodent CDRs. There may also be residues that need substituting, e.g., adding to or deleting from the human sequence. Oligonucleotides can be synthesized that can be used to mutagenize the human variable domain framework to contain the desired residues. Those oligonucleotides can be of any convenient size.

Alternatively, humanization may be achieved using the recombinant polymerase chain reaction (PCR) methodology of WO 92/07075. Using this methodology, a CDR may be spliced between the framework regions of a human antibody. In general, the technique of WO 92/07075 can be performed using a template comprising two human framework regions, AB and CD, and between them, the CDR which is to be replaced by a donor CDR. Primers A and B are used to amplify the framework region AB, and primers C and D used to amplify the framework region CD. However, the primers B and C each also contain, at their 5' ends, an additional sequence corresponding to all or at least part of the donor CDR sequence. Primers B and C overlap by a length sufficient to permit annealing of their 5' ends to each other under conditions which allow a PCR to be performed. Thus, the amplified regions AB and CD may undergo gene splicing by overlap extension to produce the humanized product in a single reaction.

In one method, humanized anti- B7 antibodies can be made by joining polynucleotides encoding portions of immunoglobulins capable of binding B7 to polynucleotides encoding appropriate human framework regions. Exemplary humanization methods can be found, e.g., in Queen et al. Proc. Natl. Acad. Sci. 1989. 86:10029 or U.S. Patent Numbers 5,585,089 or 5,693,762 the teachings of which are incorporated herein in their entirety.

In another embodiment, antibody chains or specific binding pair members can be produced by recombination between vectors comprising nucleic acid molecules encoding a fusion of a polypeptide chain of an antibody and a component of a replicable genetic display package and vectors containing nucleic acid molecules encoding a second polypeptide chain of a single binding pair member using techniques known in the art, e.g., as described in US patents 5,565,332, 5,871,907, or 5,733,743.

E. Combinatorial Anti-Costimulatory Molecule Antibodies. Both monoclonal and polyclonal antibody compositions of the invention can also be produced by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal anti-costimulatory molecule antibodies, as well as a polyclonal anti-costimulatory molecule population (Sastry et al. (1989) *PNAS* 86:5728; Huse et al. (1989) *Science* 246:1275; and Orlandi et al. (1989) *PNAS* 86:3833). After immunizing an animal with a costimulatory molecule immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) Biotechniques 11: 152-156). A similar strategy can also been used to amplify human heavy and light chain variable

regions from human antibodies (Larrick et al. (1991) *Methods: Companion to Methods in Enzymology* 2:106-110). The ability to clone human immunoglobulin V-genes takes on special significance in light of advancements in creating human antibody repertoires in transgenic animals (see, for example, Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326; and Wood et al. PCT publication WO 91/00906).

In an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combinantion, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated anti-costimulatory molecule antibody display library can be found in, for example, the Ladner et al. U.S. Patent No. 5,223,409; the Kang et al. International Publication No. WO 92/18619; the Dower et al. International Publication

No. WO 91/17271; the Winter et al. International Publication WO 92/20791; the Markland et al. International Publication No. WO 92/15679; the Breitling et al. International Publication WO 93/01288; the McCafferty et al. International Publication No. WO 92/01047; the Garrard et al. International Publication No. WO 92/09690; the Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with a costimulatory molecule can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

F. Hybridomas and Methods of Preparation. Hybridomas useful in the present invention are those characterized as having the capacity to produce a monoclonal antibody which will specifically immunoreact with a costimulatory molecule. As described below, the hybridoma cell producing anti-costimulatory molecule antibody can be directly implanted into the recipient animal in order to provide a constant source of antibody. The use of immuno-isolatory devices to encapsulate the hybridoma culture can prevent immunogenic response against the implanted cells, as well as prevent unchecked proliferation of the hybridoma cell in an immunocompromised host. A preferred hybridoma of the present invention is characterized as producing antibody

molecules that specifically immunoreact with a costimulatory molecule expressed on the cell surfaces of activated human B cells.

Methods for generating hybridomas that produce, e.g., secrete, antibody molecules having a desired immunospecificity, i.e., having the ability to bind to a particular costimulatory molecule, and/or an identifiable epitope of a costimulatory molecule, are well known in the art. Particularly applicable is the hybridoma technology described by Niman et al. (1983) *PNAS* 80:4949-4953; and by Galfre et al. (1981) *Meth. Enzymol.* 73:3-46.

In another exemplary method, transgenic mice carrying human antibody repertoires can be immunized with a human costimultory molecule. Splenocytes from these immunized transgenic mice can then be used to create hybridomas that secrete human monoclonal antibodies specifically reactive with a human costimultory molecule (see, e.g., Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT publication WO 92/03918; Kay et al. PCT publication 92/03917; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; and Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326).

The term "antibody" as used herein is intended to include fragments thereof which are also specifically reactive with a costimulatory molecule as described herein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Antibodies made using these or other methods can be tested to determine whether they inhibit a costimulatory signal in a T cell using the methods described below.

In one embodiment an antibody for use in the claimed methods binds to both B7-1 and B7-2. In making such an antibody, for example, portions of the extracellular domain which are conserved between the two costimulatory molecules can be used as the immunogen. See, e.g., Metzler et al. 1997 *Nat Struct. Biol.* 4:527).

In one embodiment, an antibody for use in the claimed methods is an antibody which binds to B7-1. Such antibodies are known in the art or can be made as set forth above using a B7-1 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Examples of B7-1 antibodies include those taught in U.S. Patent 5,747,034 and in McHugh et al. 1998. *Clin. Immunol. Immunopathol.* 87:50 or Rugtveit et al. 1997. *Clin Exp. Immunol.* 110:104.

In another embodiment, an antibody for use in the claimed methods is an antibody which binds to B7-2. Such antibodies are known in the art or can be made as set forth above using a B7-2 molecule or a portion thereof as an immunogen and screened using the methods set forth above or other standard methods. Examples of B7-2 antibodies include those taught in Rugtveit et al. 1997. *Clin Exp. Immunol.* 110:104.

In one embodiment, the claimed methods employ a combination of an antibody which binds to B7-1 and an antibody which binds to B7-2.

III. Expression of Antibodies

An antibody, or antigen binding portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989),

Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express an anti-B7 antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V_K Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference).

To express the antibodies, or antigen binding portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, can be inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the antibody-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the antibody-related VH and VL sequences to full-length antibody genes is to

insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

The nucleic acid sequences of the present invention capable of ultimately expressing the desired antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of diffcrent techniques. Joining appropriate genomic and synthetic sequences is presently the most common method of production, but CDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and Reichmann, L. et al., Nature 332, 323-327 (1988), both of which are incorporated herein by reference).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the

adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp

(1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to a B7 molecule. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than a B7 molecule by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr- CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from

the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

Antibodies, (e.g., whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention), can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

In view of the foregoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of the antibodies and antibody portions of the invention.

It will be appreciated by the skilled artisan that nucleotide sequences encoding antibodies, or portions thereof (*e.g.*, a CDR domain, such as a CDR3 domain), can be derived from the nucleotide sequences encoding the antibody using the genetic code and standard molecular biology techniques.

The invention also provides recombinant expression vectors encoding both an antibody heavy chain and an antibody light chain. For example, in one embodiment, the invention provides a recombinant expression vector encoding:

a) an antibody light chain having a variable region of an anti-B7 antibody or a humanized form thereof; and

b) an antibody heavy chain having a variable region of an anti-B7 antibody or a humanized form thereof.

The invention also provides host cells into which one or more of the recombinant expression vectors of the invention have been introduced. Preferably, the host cell is a mammalian host cell, more preferably the host cell is a CHO cell, an NS0 cell or a COS cell.

Still further the invention provides a method of synthesizing a recombinant human antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant human antibody of the invention is synthesized. The method can further comprise isolating the recombinant human antibody from the culture medium.

IV. Therapeutic Uses of Agents that Block a B7 Activity in Inhibition of Immune Responses

The agents of the current invention can be used therapeutically to inhibit immune responses through blocking receptor:ligand interactions necessary for costimulation of the T cell. Agents for use in the instant invention can be identified by their ability to inhibit T cell proliferation and/or cytokine production when added to an *in vitro* costimulation assay as described herein. The ability of such agents to inhibit T cell functions may result in immunosuppression and/or tolerance when these antibodies are administered *in vivo*.

Assays to test the blocking activity of agents for use in therapeutic applications take advantage of the functional characteristics of the B7 antigen. As previously set forth, the ability of T cells to synthesize cytokines depends not only on occupancy or cross-linking of the T cell receptor for antigen ("the primary activation signal provided by, for example anti-CD3, or phorbol ester to produce an "activated T cell"), but also on the induction of a costimulatory signal, in this case, by interaction with a B7 molecule. The binding of B7 to its natural ligand(s) on, for example, $CD28^+$ T cells, has the effect of transmitting a signal to the T cell that induces the production of increased levels of cytokines, particularly of interleukin-2, which in turn stimulates the proliferation of the T lymphocytes. Other assays for B7 function thus involve assaying for the synthesis of cytokines, such as interleukin-2, interleukin-4 or other known or unknown novel

cytokines, and/or assaying for T cell proliferation by $CD28^+$ T cells which have received a primary activation signal.

The ability of an agent to inhibit (or completely block the normal B7 costimulatory signal and induce a state of anergy) can be determined using restimulation cultures which determine the ability of T cells to be restimulated with an antigen in secondary cultures. If the T cells are unresponsive to the subsequent activation attempts, as determined by IL-2 synthesis and T cell proliferation, a state of anergy has been induced. See, e.g., Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci.* USA 90, 6586-6590; and Schwartz (1990) *Science*, 248, 1349-1356, for assay systems that can used as the basis for an assay in accordance with the present invention. The ability of an anti-B7 antibody to block or inhibit T cell costimulation is assayed by adding an agent to be tested and a primary activation signal such as antigen in association with Class II MHC to a T cell culture and assaying the culture supernatant for interleukin-2, gamma interferon, or other known or unknown cytokine. For example, any one of several conventional assays for interleukin-2 can be employed, such as the assay described in *Proc. Natl. Acad. Sci. USA*, 86:1333 (1989) which is incorporated herein by reference. A kit for an assay for the production of interferon is also available from Genzyme Corporation (Cambridge, MA.). T cell proliferation can also be measured by a assaying $[^3H]$ thymidine incorporation.

Given the agents that block a B7 activity described herein, it is possible to downregulate the function of a B7 antigen, and thereby downregulating immune responses, in a number of ways. Downregulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T cells may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both.

Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. In one embodiment, tolerance, which involves inducing non-responsiveness or anergy in T cells, is induced in a subject. Tolerance is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the

lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent.

In particular, the subject methods are useful in preventing the rejection of cell or organ transplants. Cells or organs can be transplanted from allogeneic or xenogeneic donors. Preferably, the cell or organ transplant is from an allogeneic donor.

For example, blockage of T cell function results in reduced tissue destruction in, e.g., tissue, skin and organ transplantation or in graft-versus-host disease (GVHD) transplantation. Often, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the transplant. Blocking B7-1 and B7-2 function according to the claimed methods acts as an effective immunosuppressant.

The efficacy of particular B7 blocking agent (or combination of agents, e.g., anti-B7-2 and anti-B7-1) in preventing organ transplant rejection or GVHD can be assessed using animal models that are predictive of efficacy in humans. The functionally important aspects of B7 are likely to be conserved structurally between species and therefore other B7 molecules may function across species, thereby allowing use of reagents in different species.

V. Administration of Additional Agents

In one embodiment, at least one antibody that binds to a B7 molecule can be used with other immunosuppressive agents, e.g., antibodies against other immune cell surface markers (e.g., CD40) or against cytokines, other fusion proteins, e.g., CTLA4Ig, or immunosuppressive drugs (e.g., cyclosporin A, FK506, steroids (e.g. prednisone), or rapamycin). In one embodiment of the invention the subject method comprises administering a combination of two anti-B7 antibodies and a rapamycin compound to a subject undergoing transplantation.

As used herein the term "rapamycin compound" includes the neutral tricyclic compound rapamycin, rapamycin derivatives, rapamycin analogs, and other macrolide compounds which are thought to have the same mechanism of action as rapamycin (e.g., inhibition of cytokine function). As used herein the term "rapamycin compound" includes the neutral tricyclic compound rapamycin, rapamycin derivatives, rapamycin

analogs, and other macrolide compounds which are thought to have the same mechanism of action as rapamycin (e.g., inhibition of cytokine function). The language "rapamycin compounds" includes compounds with structural similarity to rapamycin, e.g., compounds with a similar macrocyclic structure, which have modified to enhance therapeutic benefit. Exemplary rapamycin compounds suitable for use in the invention are known in the art. In addition, other methods in which rapamycin has been administered are known in the art. For example, see WO 95/22972, WO 95/16691, WO 95/04738, US 6,015,809; 5,989,591; US 5,567,709; 5,559,112; 5,530,006; 5,484,790; 5,385,908; 5,202,332; 5,162,333; 5,780,462; 5,120,727.

The language "FK 506-like compounds" includes FK 506, and FK 506 derivatives and analogs, e.g., compounds with structural similarity to FK 506, e.g., compounds with a similar macrocyclic structure, which have modified to enhance therapeutic benefit. Examples of FK506 like compounds include, for example, those described in WO 00/01385. Preferably, the language "rapamycin compound" as used herein does not include FK506-like compounds.

VI. Administration of Therapeutic Compositions

The agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to inhibit immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the agent. The term subject is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent of the invention as described herein can be in any pharmacological form including a therapeutically active amount of agent that blocks a B7 activity alone or in combination with an agent that blocks the activity of a second B7 antigen and a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessay to achieve the desired result. For example, a therapeutically active amount of an anti-B7 antibody may vary

according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. A dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The agent (e.g., antibody) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer an agent of the invention by other than parenteral administration, it may be necessary to coat the peptide with, or co-administer the agent with, a material to prevent its inactivation. An agent that blocks a B7 activity may be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Exemplary adjuvants include alum, resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition will preferably be sterile and fluid to the extent that easy syringability

exists. It will preferably be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating an agent (e.g., anti-B7 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., antibody) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the agent is suitably protected, as described above, the protein may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic

compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In one embodiment of the present invention a therapeutically effective amount of an antibody to a B7 protein is administered to a subject. As defined herein, a therapeutically effective amount of an agent (*i.e.*, an effective dosage) ranges from about 0.001 to 50 mg/kg body weight, preferably about 0.01 to 40 mg/kg body weight, more preferably about 0.1 to 30 mg/kg body weight, about 1 to 25 mg/kg, 2 to 20 mg/kg, 5 to 15 mg/kg, or 7 to 10 mg/kg body weight, e.g., in the case of an antibody. The

optimal dose of the antibody given may even vary in the same patient depending upon the time at which it is administered.

With respect to immunosuppressive compounds, appropriate doses can also be readily determined by one of ordinary skill in the art. For example, levels of CSA can range from about 150 to about 300 ng/ml. Methyl prednisolone can be administered at about 0.2 mg/kg/day to about 2mg/kg/day

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. For example, the amount of agent may remain the same or may be increased or decreased after transplantation. Changes in dosage may result from the results of assays designed to monitor transplant status (e.g., whether rejection or an immune response in the subject has occurred) as known in the art or as described herein.

In one embodiment, a pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1 to 50 mg of antibody. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980), which is incorporated herein by reference. The compositions comprising the present antibodies can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions can be administered to a patient already suffering from a disease, in an amount sufficient to

cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the clinical situation and the general state of the patient's own immune system. For example, doses for preventing transplant rejection may be lower than those given if the patient presents with clinical symptoms of rejection. Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used. It is also provided that certain protocols may allow for one or more agents describe herein to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, olyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are

dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals. The specific dose can be readily calculated by one of ordinary skill in the art, e.g., according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in assay preparations of target cells. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method for the invention, the therapeutically effective dose can be estimated initially

from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Thus, the dosage of any of the subject agents, e.g., antibodies or immunosuppressive drug can be easily determined by one of ordinary skill in the art. The dose may vary depending on the age, health and weight of the recipient, the extent of disease, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired. Exemplary doses for the anti-B7 antibodies of the invention include 3 mg/kg, 5, mg/kg, 10 mg/kg, 15 mg/kg, or 20 mg/kg. It should be noted that the dose of antibody given to one subject may vary during the course of the treatment.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. Kits for practice of the instant invention are also provided. For example, such a kit comprises an antibody reactive with B7. The kit can further comprise a means for administering the antibody conjugate, e.g., one or more syringes. The kit can come packaged with instructions for use.

VIII. Protocol of Administration

At least one agent that blocks a B7 molecule can be administered to a subject undergoing a transplant at various times relative to the transplantation procedure. In one embodiment of the invention, at least one agent that blocks a B7 molecule is administered to a subject prior to transplantation. In one embodiment, at least one agent that blocks a B7 molecule is administered between about 10 days to about 1 day prior to transplantation. In another embodiment, at least one agent that blocks a B7 molecule is administered between about 7 days to about 2 days prior to transplantation. In still another embodiment, at least one agent that blocks a B7 molecule is administered between about 5 to about 3 days prior to transplantation. In yet another embodiment, at least one agent that blocks a B7 molecule is administered about 4 days prior to transplantation. In another embodiment, a combination therapy comprising at least two

agents that blocks a B7 molecule can be administered at any of these times prior to transplantation. In another embodiment, an immunosuppressive drug is administered in conjunction with administration of one or more antibodies prior to transplantation.

In another embodiment, at least one agent that blocks a B7 molecule is administered to a subject post transplantation. For example, in one embodiment, a subject undergoing transplantation may receive at least one dose of antibody after surgery, e.g., beginning at day 2. Optionally, administration of such an antibody can continue, e.g., at weekly intervals, as deemed beneficial by one of ordinary skill in the art. In another embodiment, a combination therapy comprising at least two agents that block a B7 molecule can be administered at any of these times post transplantation. In another embodiment, an immunosuppressive drug is administered in conjunction with administration of one or more agents post transplantation.

In yet another embodiment, at least one agent that blocks a B7 molecule is administered to a subject both prior to transplantation and post transplantation. For example, the schedule of administration prior to transplantation outlined above and the schedule of administration post transplantation outlined above can be combined. In another embodiment, a combination therapy can be administered using such a schedule of pre- and post- transplantation administration. In another embodiment, an immunosuppressive drug is administered in conjunction with administration of one or more antibodies prior to and post transplantation.

For example, in one embodiment, at least one agent that blocks a B7 molecule is administered prior to surgery (e.g., from about four days prior to immediately prior) and then again after surgery. In a preferred embodiment of the invention, at least one agent that blocks a B7 molecule is administered immediately following anesthesia induction and again and immediately following surgery. In another embodiment, at least one agent that blocks a B7 molecule is administered at least about 1, 3, 5, 10, or 20 hours to a number of days (e.g., between 1-10 days) prior to surgery and then again at least about 1, 3, 5, 10 or 20 hours to a number of days (e.g., between 1-10 days) after surgery. In yet another embodiment one or more agents that block a B7 molecule can also be administered during the surgical procedure. In another embodiment, a combination therapy can be administered using any of these protocols. In another embodiment, an

immunosuppressive drug is administered in conjunction with administration of one or more antibodies according to any of these protocols.

As set forth above, in a preferred embodiment, additional agents, e.g., immunosuppressive drugs (such as Rapamycin compounds, steroids such as prednisone, FK506, or cyclosporin A) can be administered using any protocol of choice in conjunction with any one of the above protocols for administration of at least one agent that blocks a B7 molecule. For example, in one embodiment, an immunosuppressive agent can be administered prior to transplantation. In another embodiment, an immunosuppressive agent can be administered post transplantation. In yet another embodiment, an immunosuppressive agent can be administered both prior to and post transplantation.

The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Example 1 Administration of Anti-B7 Antibodies Prior to Transplantation Promotes Graft Survival

Unilateral renal transplantation was performed in 12 blood group matched and mixed lymphocyte reaction (MLR) mismatched animals. All monkeys were treated with the combination of humanized B7-1 (h1F1) and B7-2 (h3D1) monoclonal antibodies (mAb) given according to three distinct schedules. Group 1 (n=4) received h1F1 (20mg/kg) and h3D1 (20 mg/kg) immediately following anesthesia induction and 5 mg/kg of each mAb at the end of surgery. In group 2 (n=4) h1F1 and h3D1 were administered immediately before surgery (5mg/kg each) and then 10 mg/kg of each mAb was administered at the end of surgery. This group received an additional dose (10

mg/kg each) of both mAb on day 3. In group 3, the schedule described for group 1 was used and an extra 10 mg/kg of both antibodies were administered 4 days prior to surgery. All groups then received a dose of 5 mg/kg of each mAb in weekly intervals starting at day 7 and ending at day 56. The animals were then observed for a maximum of 119 days.

The 4 animals of group 1 survived 9, 119, 119, and 48 days (mean 74 days) versus 18, 119, 14, and 12 days in group 2 (mean 41 days). Three of the 4 animals are still alive in group 3, with a present mean survival of >71 days. Between day 4 and day 8, all animals experienced clinically diagnosed acute rejection episodes of varying severity. In group 1 repeated scheduled administration was sufficient to reverse severe rejection in 3 of 4 animals. Only one animal in group 2 was able to recover from early severe rejection. In group 3, 3 of 4 animals showed mild clinical rejection that was easily reversed, while one animal was sacrificed at day 10 due to terminal rejection.

The current study demonstrates that repeated administration of h1F1 and h3D1 according to the schedule for group 1 is sufficient to provide long-term graft survival in 75% of the animals studied, 2 of which survived more than 60 days following termination of immunosuppressive therapy. While the purpose of the schedule used in group 2 was to overcome the early rejection episodes observed in group 1 by adding an additional dose of mAb on day 3, this latter schedule in the end proved less effective, most likely owing to the lower loading doses during surgery. Preloading the animals with MAb 4 days prior to surgery resulted in less severe rejection with excellent long-term outcome in the majority of the recipients. This demonstrates for the first time how critical timing and dosing is for these otherwise very potent immunosuppressive agents.

Example 2. Administration of Sirolimus Enhances Transplant Survival.

Unilateral renal transplantation was performed in 12 blood group matched and MLR mismatched animals. Group 1 (n=4) received mAb therapy; group 2 (n=4) received mAb plus sirolimus; group 3 (n=4) received sirolimus monotherapy. mAb therapy consisted of the combination of a B7-1 (h1F1) and B7 (h3D1) mAb given at 20 mg/kg each preoperatively, then 5 mg/kg each in weekly intervals starting at day 0 until day 56 (9 doses). Sirolimus was administered by daily gavage at 1 mg/kg at day 0 to

day 13 and then 0.5 mg/kg from day 13 to day 56. All animals were off immunosuppression after day 56 and were then followed until day 119.

All animals in group 1 showed clinical evidence of acute severe rejection (fever, creatinine increase, anuria) within the first week post transplant. Early clinical acute rejection was only observed in 2 of the 4 animals of group 2 as was less severe and easily reversible. In group 3 acute rejection was diagnosed in all animals and was also less severe than in group 1. The mean survival of the 3 groups was 74 (group 1; range 9-119 days), 86 (group 2; 69-119 days) and 23 days (group 3; 11 to 35; one way ANOVA p=n.s.). Final histology showed signs of both acute and chronic rejection in all long-term survivors of group 1 through 3. The sirolimus trough levels in group 2 and 3 were between 10 and 20 ng/ml from day 0 to day 13 and then between 5 and 10 ng/ml until day 56.

These data show for the first time in monkey allograft recipients that combining sirolimus with mAb directed against costimulaotry signals can improve graft outcome and lessen the incidence and severity of early acute rejection.

The following materials and methods were used in Examples 3-6

Animals These studies were approved by the Institutional Animal Care and Use Committee at Stanford University, a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and registered with the United States Department of Agriculture. Male, wild caught, cynomolgus monkeys, *Macaca fascicularis,* with a weight between 5 and 8 kg were obtained from Charles River Biomedical Research Foundation, Inc. (Houston, Texas, USA). The animals underwent blood group typing by the New York University Medical Center Laboratory for Experimental Medicine and Surgery in Primates. Donor and recipient monkeys were paired based on an ABO blood group match, a negative cross-match, and a stimulation index of at least 2.5 in a two-way mixed lymphocyte reaction assay. The animals received humane care in compliance with the "Principals of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and

published by the National Institutes of Health (NIH Publication No.80-123, revised 1985).

Drugs and Treatments Individual humanized monoclonal antibodies against the human CD80 (hlFl) or the CD86 (h3D1) receptor were obtained from Genetics Institute/Wyeth Aerst, Inc. (Cambridge, MA) in two different batches. Both hlF1 and h3D1 were drawn into a single syringe and then given via a syringe pump at a maximum infusion rate of 1 mg/kg/min through a peripheral venous catheter. Microemulsion formulations of cyclosporine (Neoral) for oral administration were purchased from Novartis Pharmaceuticals (East Hanover, NJ).The CsA microemulsion (100 mg/ml) was kept at room temperature. The calculated volume of Neoral based on the required dose (in mg/kg) was drawn directly into the smallest appropriate syringe and administered without any dilution through a nasogastric tube directly into the stomach. This tube was rinsed with 20 mL of water. Methylprednisolone (Solu-Medrol, Pharmacia & Upjohn Co., Kalamazoo, MI) was supplied in vials with self-contained sterile water for reconstitution according to manufacturer's instructions. The drug was kept refrigerated after reconstitution and discarded after 48 hours

Prednisone (Mutual Pharmaceutical Co., Inc., Philadelphia, PA) was supplied in tablets (5 mg). Tablets were dissolved in sterile water, one tablet per 5 mL water, with a resulting solution concentration of 1 mg/mL. The remaining drug was discarded at the end of dosing each day. Both prednisone and microemulsion cyclosporine were administered once daily by transoral gavage in the sedated animal

Pharmacokinetic monitoring

Prior to each antibody administration, a serum sample was collected to determine pre-administration antibody levels. A second serum sample was collected upon completion of antibody administration by puncture of a femoral vein opposite to the injection site. These serum samples were frozen for later assessment. Twenty-four hour trough levels of CsA were measured three times per week and the daily CsA dose was adjusted to meet target CsA trough levels (see below). CsA was quantified using the validated

LC-MS assay as previously described by Christians et al. Additional modifications of the CsA dose were performed to prevent excessive weight loss or when renal functional impairment was thought to be related to CsA trough levels.

Experimental groups

In group I, hlF1 (CD80 mAb) and h3D1 (CD86 mAb) were administered at a dose of 20 mg/kg each after sedation of the animals immediately before surgery. The animals then received additional doses of both mAb at 5mg/kg each starting immediately postoperatively and then every 7 days until poday (post-operative day) 56. In group II the same antibody administration schedule was followed. In addition, animals in this group received a daily dose of CsA adjusted to maintain 24 hour through levels between 200 and 300 ng/ml for podays I to I3 and then I50 to 250 ng/ml for podays 14 to 56. All immunosuppressive therapy was discontinued on poday 56. Animals in group III received CsA adjusted daily to obtain the same target trough levels as outlined for group II without receiving the monoclonal antibodies. Group IV was treated with the same mAb schedule as group I and II. In addition, animals in group IV received methylprednisolone at a dose of 2 mg/kg/d given intravenously as a bolus for the first 3 postoperative days (poday 0 through 2), then prednisone by oral gavage at a dose starting at 0.5 mg/kg/d, which was tapered by 0.05 mg/kg every 3 days until a dose of 0.2 mg/kg/d was reached. This dose of prednisone was maintained until poday 56, after which time all immunosuppressive therapy was discontinued.

Life-supporting unilateral renal transplantation

Animals in groups I and II were sedated with ketamine 10 mg/kg intramuscularly and anesthesia was maintained with isoflurane (.5 to 2% Vol,AErrane, Ohmeda Inc., Liberty Corner, NJ). Due to isoflurane induced hypotension, animals in later groups received a loading dose of midazolarn 0.1 mg/kg IV (Roche Pharmaceuticals, Nutley, N.J.) followed by a bolus of propofol (Abbott Labortatories, North Chicago, IL) given to effect for intubation. Once intubated, animals were maintained on constant infusions of propofol 0. lmg/kg/min and midazolam 0.35 ug/kg/min.. The animals were left to

breathe spontaneously without ventilator assist, but still received oxygen at 1.5-2.0 L/min through the endotracheal tube.

For the transplant procedure, a recipient monkey, that had been used as a donor previously, and a naive kidney donor were anaesthetized at the same time. The left kidney was harvested from the donor animal following partial clamping of the aorta and in-situ flush perfusion of the kidney with 20 ml of cold Euro Collins solution. The donor kidney was then implanted into the lower abdomen of the recipient animal by end-to-side anastomosis of the renal artery and vein to the recipient aorta and vena cava, respectively. A telemetric pressure probe was inserted into the recipient aorta to continuously monitor systemic arterial pressure, heart rate and core temperature of the animal during the entire follow-up. The ureter was tunneled through the bladder wall and attached to the bladder mucosa with a single PDS 5-0 stitch. Animals were allowed to recover overnight in an intensive care unit and 30 ml/kg of Lactated Ringer's solution was administered intravenously 4, 8 and 16 hours postoperatively. Buprenorphine was given at a dose of 0.01-0.03 mg/kg intramuscualarly every 8-12 hrs for pain. For hydration the animals were offered only water after POD 0 and after that had free access to food and water.

Follow-up Urine output and fluid intake were recorded daily for the first 14 postoperative days. Vital signs, appetite, attitude and amount and consistency of feces were recorded daily during the entire postoperative period. After initial visual assessment, animals were sedated for treatments or blood draws or both with ketamine in a dose range of 5-10 mg/kg IM. Upon sedation, animals were weighed and assessed for hydration status and wound status. Detailed clinical observations were performed 3 times per week during the first week and then once weekly for the remainder of the follow-up. Animals were euthanized if there was severe oliguria or anuria and the creatinine rose above 7 mg/dl on more than two consecutive days. In addition, animals were sacrificed if weight loss was in excess of 25% when compared to the animal s weight at the time of surgery.

Biopsies and histology For the animals in group II though IV, percutaneous renal biopsies were taken on postoperative days 7, 28 and 70. At the time of sacrifice, animals were euthanized with 3 mg of pentobarbital sodium. The transplanted kidney was removed and stored in 10 % neutral buffered formalin. Sections were prepared and stained with hematoxylin and eosin. Pathologists blinded to the individual treatment groups evaluated the resulting slides based on the modified Banffcriteria (Racusen LC, et al.. Kidney Int 1999; 55: 713).

Statistics Statistical analyses were performed using the statistics program SPSS for Windows, version 10.07 (SPSS Inc., Chicago, Illinois). Values are shown as means ± standard error of the mean for parametric data and as medians for non-parametric data. For repeatedly assessed parametric data (animal weight), groups were compared using the repeated measure ANOVA test in combination with the Bonferoni post-hoc test. One-way-analysis of variance in combination with post hoc tests was used for non-repeated measures of parametric data. Animal survival was analyzed by a Kaplan Meier test and groups compared with a Log rank analysis. A p-value equal to, or less than, 0.05 was considered as significant.

Example 3. Demographics of the transplant groups

The recipient animal weights ranged from 4.75 kg to 7.75 kg without a significant difference among the 4 experimental groups (Table 1). The minimum stimulation index measured by mixed lymphocyte reaction was 2.5 with an average stimulation index 13 ± 5.2 in group I (mean ± standard error of mean), 6.8 ± 1.7 in group II, 11.6 ± 5.3 in group III and 13.5 ± 4.6 in group IV. The graft ischemic intervals ranged from 43 to 83 minutes with shorter ischemic intervals occurring more frequently in later experimental groups. The mean ischemic interval of group III (49 ± 2 minutes) was significantly shorter than in group I (66 ± .6 minutes; $p < 0.02$).

Example 4. Pharmacokinetic data

The average plasma trough levels of h3D1 and hIF1 are depicted in Figure 1. There was no significant difference in the h3D1 and hIF1 trough levels over time by

repeated measures analysis (p = .713 and p = .272, respectively). The curves show a slow decline of plasma antibody levels after cessation of mAb administration on poday 56. This linear decline in plasma levels rules out neutralization of the infused monoclonals by primate anti-human antibody formation.

The significant interindividual variation in CsA bioavailability resulted in differences in doses and trough levels during the first week post transplantation for animals in group II and III. Doses were more easily corrected and anticipated later in the postoperative course (Figure 2). The repeated measures analysis of both CsA dose and 24 hour CsA trough levels for the entire treatment period did not demonstrate a significant difference between the two groups treated with CsA (groups II and III).

Example 5. Postoperative clinical course

None of the recipient animals required euthanasia due to technical complications. One animal in group I (# 98134) had a leak in the ureter anastomosis, which was successfully corrected poday 6 and its further postoperative course was unremarkable. Evidence of ischemia - reperfusion injury was observed in some animals as shown by a substantial increase in the serum creatinine on the first postoperative day (see table 2), which. in individual animals, was accompanied by transient oliguria or anuria. All animals demonstrated a significant decline in their body weight during the first 21 postoperative days (Figure 4). The four animals in group IV showed most rapid recovery of weight loss. However, the repeated measures analysis did not show any significant difference

between the four groups (p=0.18).

Daily telemetric assessment of animal core temperature revealed a distinct pattern in many of the transplant recipients. As depicted in Figure 5, 100% of the animals of group I (mAb monotherapy) and 50% of the animals in group III (CsA monotherapy) had a febrile episode with a body temperature of more than 39°Celsius between postoperative day 5 and 7, while these febrile events were not observed in any of the animals in group II (mAb plus CsA) or group IV (mAb plus prednisone). These febrile events were generally followed by a significant rise in the serum creatinine (see table 2). Therefore, fever were believed to be due to acute rejection episodes. In three of

the four animals in group I, the repeated scheduled administration of both humanized monoclonal antibodies on postoperative day 7 was followed by a reversal of this clinical rejection episode. In one of the animals (# 98133) the injury to the allograft was substantial and the creatinine levels never returned to normal. Increases in the serum creatinine levels within the first 10 postoperative days were not observed in any of the animals of group II and III.

Example 6. Animal survival, incidence of clinical and biopsy proven rejection

Figure 6 depicts the occurrence of clinical rejection, outcome of serial percutaneous biopsies, and final histological diagnosis for individual animals. In group I (mAb treatment alone), all monkeys experienced early severe acute rejection, which was terminal in one of the monkeys (#67485). Two of the remaining three animals in this group experienced at least one more clinically diagnosed acute severe rejection episode. Despite two severe and one mild clinical rejection episode in the first 60 postoperative days, monkey # 98135 survived the entire follow up period of 120 days, the last 66 days of which no immunosuppressive agents were administered. The histology of these two animals on day 120 showed borderline changes due to rejection in one monkey and a Type IB rejection in the other. Table 3 shows that samples from allografts obtained following sacrifice showed histological features of mild chronic rejection.

In contrast to group I, none of the monkeys in group II (mAb plus CsA) experienced severe or moderate clinical rejection early in the postoperative course. Except for one biopsy in a single animal, which showed a Type IA rejection, all other biopsies taken on poday 7, 28 and 70 (n=11) were considered normal. Once all the immunosuppressive therapy was discontinued on poday 56, three of the 4 animals in this group experienced either a moderate or a severe acute clinical rejection. Three of the 4 animals in this group survived 120 days, all of which had histological features of acute rejection without evidence of chronic rejection.

In group III, immunosuppressive therapy consisted of CsA monotherapy. This type of immunosuppression less effectively prevented early acute clinical rejection. Within the first 21 postoperative days, all monkeys in this group had either severe acute clinical rejection or biopsy proven rejection. With the reduction in the CsA target trough levels

after poday 15, the incidence of acute rejection increased and finally 3 of the 4 monkeys in this group were euthanized early due to acute rejection. The one longer surviving monkey was euthanized on poday 71 with histological features of a Type IB rejection, a serum creatinine of 11.7 mg/dl and significant weight loss. Log rank analysis in the Kaplan - Meier test demonstrated that the survival times of group III were significantly shorter than the survival times in group II ($p<0.014$).

Co-administration of mAb plus initial methylprednisolone, followed by prednisone until poday 56 (Group IV), reduced the incidence and severity of acute clinical rejection. Only one animal in this group had early acute clinical rejection and was euthanized on poday 6 (#2568). Two monkeys of this group showed evidence of either clinical or biopsy proven rejection at postoperative days 8 and 28. One of these two animals survived the entire follow-up period and the allograft samples obtained following euthanasia showed histological features of Type 1B rejection without evidence of chronic rejection. One animal, that was sacrificed on poday 77 due to terminal acute rejection, had significant chronic alterations in the samples obtained at necropsy.

These Examples demonstrate that after life-supporting renal transplantation the combined treatment with humanized mAb directed against CD80 and CD86 results in longterm animal survival in the majority of the animals, which was significantly better than observed in untreated historical controls (mean survival 8 days). Despite the presence of long - term survival following B7 antibody administration alone, graft tolerance was not observed. The nonhuman primates studied did not develop neutralizing antibodies and repeated administration of both mAb resulted in trough levels in excess of 100 μg/ml for many days post cessation of treatment. The antibody was well tolerated without signs of anaphylaxis or thrombotic complications. Based on the efficacy data of B7 mAb treatment alone, this combination of mAb treatment can be recommended as induction therapy in solid organ transplantation. The efficacy of these mAb was further improved by co-administration of microemulsion cyclosporine, although the difference in animal survival between mAb therapy alone and combined treatment with CsA was not significant. However, combined treatment of CsA plus

anti-B7 mAb resulted in significantly longer survival than CsA monotherapy. Adding CsA or steroids to the mAb did not antagonize efficacy of the mAb.

Previous preclinical research in non-human primates with antibodies directed against the CD80 and CD86 receptors has been limited to studies conducted by Ossevoort et al (Ossevoort MA, et al. Transplantation 1999; 68: 1010) using murine CD80 and CD86 mAb and Kirk et al using both murine and humanized monoclonal antibodies directed against the CD80 and CD86 receptors (Kirk AD, et al. Nat Med 1999; 5: 686). In a skin allograft model in rhesus monkeys using a murine mAb against CD80 in combination with a sub-therapeutic dose of CsA, Ossevoort was able to extend allograft survival from 5 (untreated controls) to 14 days (Ossevoort MA, Transplant Proc 1998; 30: 1061).When combining the murine monoclonals against CD80 and CD86 in life supporting renal transplantation in the same animal species, Ossevoort and colleagues report a mean survival of 28 $\pm$ 7 days in 4 animals compared to an average survival of 6 days in untreated controls (Ossevoort MA, et al. Transplant Proc 1998; 30: 2165; Ossevoort MA, et al. Transplantation 1999; 68: 1010). The combination of both monoclonals with CsA given at 10 mg/kg IM was similar to the survival following CsA treatment alone. The efficacy of the murine monoclonals was limited by the early development of primate anti-murine antibodies. Kirk et al have performed experiments in life supporting renal transplantation in rhesus monkeys with both murine and humanized anti CD80 and anti CD86 monoclonal antibodies(Kirk AD. Crit Rev Immunol 1999; 19: 349; Kirk AD, et al. Transplantation 2000; 64: S7; Kirk AD, et al. Transplantation 2000;66: S6).

Administration of either the murine anti CD80 or anti CD86 mAb individually resulted in only modest prolongation of graft survival (anti-CD80: 36 and 40 days; anti-CD86: 9 and 13 days). Co-administration of both murine antibodies resulted in graft survival times of 25, 42, 77 and 227 days (Kirk AD, et al. Transplantation 2000;69: S414). Administration of humanized mAb to the CD80 or the CD86 receptor resulted in 8 and 9 day survival for animals treated with the anti CD80 mAb and 8 and 28 day survival for those animals treated with the anti CD86 mAb. When both humanized antibodies were combined and administered using a protocol very similar to the one used in the current study, the survival times for 4 individual animals were as follows: 47,

67, 227, and > 407 (animal still alive)(Kirk AD, Tadaki DK, Xu H et al. Transplantation 2000;69: S414).

Data derived from these non-human primate experiments paralleled data obtained from mouse transplantation studies. In murine experiments, co-administration of mAb directed against the CD80 and CD86 receptors have also proven significantly more effective than administration of either mAb alone. However, in respect to the induction of long-term tolerance, the murine data are inconsistent. In studies conducted by Woodward et al (Woodward JE, Bayer AL, Chavin KD, Blue ML, Baliga P. T-cell alterations in cardiac allograft recipients after B7 (CD80 and CD86) blockade. Transplantation 1998;1998 Jul 15; 66: 14) and Lenschow et al (Lenschow DJ, Zeng Y, Hathcock KS et al. Transplantation 1995;1995 Nov 27; 60: 1171) tolerance was not induced. These data contradict results reported by Bashuda et al (Bashuda H, et al. Transplant Proc 1996; 28: 1039), where tolerance was induced. Interestingly, both Woodward and Bashuda used the same rat-anti-mouse antibody at the same dose, in the same organ transplant model (heart) with slightly different mouse strain combinations.

Based on the current literature on the use of anti-CD80 and anti-CD86 antibodies in solid organ transplant models and based on the results presented here, where a high dose induction protocol followed by weekly lower doses of both antibodies was used, anti B7 mAb do not consistently induce long-term tolerance.

These data show that anti-CD80 and anti-CD86 mAb therapy alone could not prevent early severe acute rejection, which was terminal within 10 days of transplantation in one of the 4 animals in the mAb alone group, and resulted in severe renal dysfunction with highly elevated serum creatinines in the remaining three monkeys in this group. This is a new finding, which has not been described in any of the murine or non-human primate experiments to date.

After the first dose of anti-CD80 and anti-CD86 mAb, plasma mAb trough levels were significantly above the range (> 100 ug/ml), which has been shown to saturate CD80 and CD86 receptors on PBMCs in non-transplanted animals. Based on known antibody levels in the central compartment (plasma), and known elimination rates of these antibodies, which can be calculated using the concentration profile and the rate of elimination in the terminal (post-distribution) phase, an estimation of the average

antibody concentration in secondary compartments (all tissues other than blood) can be performed. The estimated average concentration of anti-CD80 and anti-CD86 in all tissues outside the blood was approximately 6.5 mg/mL over the first 7 days post transplant. Accurate estimates of concentrations within individual tissues that may be more critical than others, in terms of hosting costimulatory interactions, is not possible using the data collected in this study. Therefore, it is unknown whether the lack of efficacy observed early post transplantation is due to the lack of inhibition of the CD80 / CD86 to CD28 interaction in tissues outside the blood.

The lack of a primary antibody response to the humanized anti B7 monoclonals (primate antihuman antibody response; PAHA) is not shared by most other humanized monoclonals evaluated in non human primate models (Poston RS, Robbins RC, Chan B et al. Transplantation 2000;69: 2005.). These data support the hypothesis that the B7 monoclonal antibodies successfully block the T and B cell interaction necessary to elicit an antibody response.

As described previously, the CD80 and CD86 receptors expressed on antigen presenting cells interact with both CD28 and CTLA-4 on T cells. This dual specificity of the B7 co-stimulatory molecules has made it challenging to elucidate the functions of this key immunoregulatory pathway. There is recent evidence that T -cell anergy is primarily induced not by the absence of B7 - CD28 interaction but as a result of B7 - CTLA-4 interactions (Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB. Immunity 2000;6: 411.). In vitro studies on the binding avidity and binding kinetics of CD80 / CD86 to CD28 and CTLA-4 receptors have shown that CTLA-4 is a high avidity receptor for both CD80 and CD86, while CD28 is a low avidity receptor (Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Immunity 1994; 1: 793.). These data suggest that at low concentrations, the B7 molecules would more favorably bind to CTLA-4 rather than CD28. Therefore, B7 antagonists may act either by blocking B7-CD28 interaction, or by reducing the effective B7 concentrations to low levels, favoring inhibitory B7 - CTLA-4 interactions. Linsley et al. have also shown that CTLA-4 may be expressed at functionally significant levels much earlier than at the time of its peak expression at 1-2 days post activation, suggesting that CTLA-4 may have an important role in regulating both the initiation and termination of T-cell responses

(Linsley PS, Bradshaw J, Greene J, Peach R, Bennett KL, Mittler RS. Immunity 2000;4: 535.). Therefore, it is possible that high concentrations of B7 mAb early after transplantation may have an inhibitory effect on the CTLA-4 mediated signal, which may further enhance the likelihood of rejection.

High concentrations of the anti-B7 molecules starting immediately at the time of transplantation and then sustained by repeated administration for the first 2 postoperative months, as following in the current study, may therefore not only inhibit the CD80/CD86 - CD28 interaction, but also inhibit the engagement of the B7 molecules with CTLA-4 and thus prevent the inhibitory signal that would allow T cells to become anergic. The complex data on avidity, binding kinetics and times of maximal receptor expression of CTLA-4 and CD28 suggest a very delicate state of interactions that may be upset by high concentrations of anti-CD80 and anti-CD86 mAb. One could hypothesize, that the overall effect of aggressive early post transplantation inhibition of the interaction of both CTLA-4 and CD28 with the CD80/CD86 receptors would be a reduction of the net inhibitory signal resulting in clonal T cell expansion and acute early rejection. In animals that survive this early rejection, further administration of lower doses of CD80/CD86 mAb may allow a stronger inhibitory signal by reducing expression of accessible CD80/CD86 receptors to a degree that would inhibit their interaction with CD28 but still have sufficient expression to interact with CTLA-4. CTLA-4 expression may be upregulated over time as a possible compensatory mechanism.

Recently a third member of the B7 family has been identified by Dong et al and has been termed B7-H1 (Dong H, Zhu G, Tamada K, Chen L. Nat Med 1999;5: 1365.). It is currently unknown if the humanized monoclonals used in the current study can block the interaction of B7-H1 and CD28 and the potential inability to do so could explain the lack of long term tolerance and the high incidence of early acute rejection. Also would could explain differences in efficacy of the B7 mAb in different strains of non human primates (i.e.: rhesus versus cynomolgus) by the fact that these different strains may be different in terms of the diversity and/or dominance of the members of the B7 family.

Multiple publications have stressed the negative impact of calcineurin inhibition or steroid administration on co-stimulation blockade induced tolerance or long-term graft survival in solid organ transplantation (Kirk AD, et al. Nat Med 1999;5: 686; Harlan DM, Kirk AD. JAMA 1999;282: 1076). Most of these data have been derived from studies evaluating CD154 monoclonal antibodies(Kirk AD. Crit Rev Immunol 1999; 19: 349.; Li Y, Zheng XX, Li XC, Z and MS, Strom TB. Transplantation 1998;66: 1387.). While the initial reports on the use of this mAb in nonhuman primates have emphasized that anti CD40L antibodies do induce tolerance, more recent presentations have stressed this mAb produces long-term graft survival rather than tolerance (Kirk AD, Tadaki DK, Xu H et al. Transplantation 2000;69: S414.). Kirk et al. have reported that co-administration of cyclosporine, tacrolimus, or steroids reduced the efficacy of the CD40L antibodies during the first 6 months post transplantation, a period during which the monoclonal antibodies were given in monthly intervals (Kirk AD, et al.. Nat Med 1999;5: 686; Kirk AD. Crit Rev Immunol 1999; 19: 349). Based on this most recent data presented by Kirk et al. the question, whether or not calcineurin inhibitors or steroids interfere with tolerance induction, may have become obsolete. Co-stimulation blockade would then have to be considered an induction therapy to facilitate long term survival when given in addition to conventional small molecule immunosuppressive agents. The question would be, what conventional immunosuppressive agents can be co-administered with costimulation blocking agents, such as the anti-CD80 and anti-CD86 antibodies used in our study. In this respect, our data clearly show clinical potentiation rather than antagonism of the immunosuppressive interaction anti-B7 antibodies and microemulsion CsA. Our data establishes the potential benefits of anti CD80/86 mAb as adjuncts to current standard immunosuppressive regimens in clinical renal transplantation. Thus, our data demonstrated that co-administration of B7 antibodies and CsA provides significantly better animal survival than CsA monotherapy.

In conclusion, these examples provide evidence for significant immunosuppressive efficacy of the novel humanized monoclonal antibodies directed against the CD80 and CD86 receptors. These agents are effective when given alone and their efficacy can be improved when administered in combination with microemulsion cyclosporine or prednisone. These Examples show that monotherapy with B7 antibodies

was associated with a high incidence of severe rejection very early in the postoperative course, which was attenuated by co-administration of either CsA or prednisone. B7 antibodies likely interfere with the inhibitory signal mediated by binding of CD80/ CD86 to CTLA4 and that this inhibitory signal may be essential for avoiding early rejection. Studies are currently ongoing to evaluate whether changes in the dose and administration schedule of B7 antibodies can reduce the incidence and severity of early acute rejection following B7 antibody monotherapy. Despite a high and prolonged B7 mAb exposure of the non-human primates there was no secondary antibody response, no anaphylaxis and no incidence of a thromboembolic event.

Table 1: Demographics

Group	Monkey ID	Group Definition	Recipient Weight	Donor Weight	Recipient Blood Group	Donor Blood Group	MLR SI	Ischemic Interval (min)	Survival (POD)
I	67485	mAb	6.40	7.05	AB	B	4.00	64	9
I	98135	mAb	5.70	7.05	AB	A	4.98	63	119
I	98134	mAb	6.85	7.15	AB	AB	17.55	83	119
I	98133	mAb	6.50	7.05	AB	AB	25.65	55	48
II	118572	mAb+CsA	7.50	7.45	B	B	4.95	60	119
II	118104	mAb+CsA	7.25	8.30	A	A	6.47	58	119
II	26117	mAb+CsA	7.70	5.94	B	O	11.73	54	119
II	126168	mAb+CsA	6.80	5.94	A	O	3.94	57	96
III	490012	CsA	5.50	6.70	B	B	6.53	48	71
III	490011	CsA	6.20	6.30	B	B	9.96	43	22
III	490055	CsA	7.00	7.10	B	B	16.71	49	38
III	590029	CsA	6.70	6.00	B	B	29.97	50	25
IV	2568	mAb+Pred	7.45	7.00	B	B	26.76	59	6
IV	118573	mAb+Pred	4.75	5.80	B	B	7.39	57	111
IV	106284	mAb+Pred	5.94	6.30	O	O	9.58	59	77
IV	490231	mAb+Pred	6.80	6.30	B	O	2.5	50	120

Abbreviations: ID = identifier, MLR SI = Mixed Lymphocyte reaction stimulation index, POD = postoperative day, mAb = monoclonal antibodies, ischemic interval in minutes

Table 2: Serum creatinine levels of individual monkeys expressed in mg/dL over the observation period. Increased creatinine levels after postoperative day 3 were interpreted as clinical evidence of acute allograft rejection

	Group I (mAb alone)				Group II (mAb + CsA)				Group III (CsA alone)				Group IV (mAb + prednisone)			
Postop Day	67485	98133	98134	98135	126168	26177	118184	118572	490011	490012	490055	590029	2568	106284	118573	490231
1	2.1	4.7	2.2	2.1	3.9	2.1	2.3	2.9	2.4	2.0	2.5	2.1	2.8	1.6	1.8	2.6
3	11.8	3.0	9.2	6.4	2.6	1.9	1.8	1.9	2.8	2.0	1.9	1.9	5.7	1.5	8.3	2.3
5	3.2	2.6	12.4	6.5	2.0	1.6	1.5	1.5	1.8	1.5	1.8	1.5	2.4	1.4	2.2	1.5
7	5.7	8.2	5.6	3.0	1.9	1.7	1.7	1.5	1.7	1.5	1.6	1.5		1.6	1.0	1.5
9	7.6	10.5	5.8			1.4		1.7		1.5	1.6	1.9			1.0	
11		10.3	4.7	1.8	4.3		1.9		1.6			2.3		3.7		
14		5.5	2.1	2.1	2.9	1.3	2.1	1.7	1.7	1.7	1.6	2.7		4.3	1.1	3.3
18		4.0	1.9	2.1	2.8		1.8	1.5	1.8	1.7	1.7	2.7		2.0	1.0	4.0
21					2.8	1.2	1.8	2.3	4.9	2.0	1.8			2.7	0.9	2.5
24		7.1	1.7	2.0	2.3	1.2	1.6	1.8	8.3	2.3	1.9	5.0		2.3	0.9	2.0
28		4.9	1.8	2.0	2.3	1.1	1.5	1.5		1.6	2.0			1.9	0.9	1.7
35		3.9	1.4	2.4	2.3	1.5	1.5	1.4		1.5	3.7			1.5	0.9	1.5
42		4.2	1.5	3.5	2.4	1.5	1.5	1.2		1.7	6.6			1.4	1.0	1.4
49		6.2	1.4	3.5	2.6	1.9	1.6	1.3		1.7				1.6	0.8	1.3
56			1.5	3.5	2.2	2.0	1.8	1.1		1.7				1.4	0.9	1.2
63			1.4	2.9	2.0	2.1	1.6	1.3		2.0				3.2	0.8	1.2
70			1.4	3.0	2.0	1.7	1.9	1.4		7.6				3.9	0.9	1.3
77			0.8	3.3	1.7	1.8		1.9		11.7				21.8	0.9	1.2
84			1.1	2.9	1.8	3.5	1.6	1.5							1.0	1.3
91			1.2	2.2	6.9	3.2	2.0	1.6							1.1	1.3
98			1.1	2.1		2.0	2.2	1.5							1.0	1.3
105			1.2	2.0		2.0	2.2	1.6							1.0	1.4
112			1.1	1.8		2.6	2.3	1.4							8.6	1.6
119			1.1	2.2		4.7	8.4	1.6								2.3

5

Table 3: Histological grading at the time of sacrifice based on the Banff 97 working classification of renal allograft pathology.

Group	Monkey ID	Group Definition	Survival (POD)	Diagnostic Category	Acute Score	Chronic Score
I	67485	mAb	9	Type IIA rejection	t2 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
I	98135	mAb	119	Type IB rejection	t3 v0 i3 g1	ci0 ct0 cg0 mm0 cv0 ah0
I	98134	mAb	119	Borderline	t1 v0 i3 g0	ci1 ct1 cg0 mm0 cv0 ah0
I	98133	mAb	48	Type III rejection	t3 v3 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
II	118572	mAb+CsA	119	Type IA rejection	t3 v3 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
II	118104	mAb+CsA	119	Type IIA rejection	t3 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
II	26117	mAb+CsA	119	Type IA rejection	t3 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
II	126168	mAb+CsA	96	Type III rejection	t3 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
III	490012	CsA	71	Type IB rejection	t3 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
III	490011	CsA	22	Type III rejection	t3 v3 i3 g1	ci0 ct0 cg0 mm0 cv0 ah0
III	490055	CsA	38	Type III rejection	t2 v3 i3 g3	ci1 ct1 cg0 mm0 cv0 ah0
III	590029	CsA	25	Type IB rejection	t3 v0 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
IV	2568	mAb+Pred	6	Type IIA rejection	t1 v0 i2 g0	ci0 ct0 cg0 mm0 cv0 ah0
IV	118573	mAb+Pred	111	Type III rejection	t3 v3 i3 g3	ci0 ct0 cg0 mm0 cv0 ah0
IV	106284	mAb+Pred	77	Type III rejection	t1 v0 i3 g0	ci3 ct3 cg0 mm0 cv0 ah0
IV	490231	mAb+Pred	120	Type IA rejection	t3 v0 i2 g3	ci0 ct0 cg0 mm0 cv0 ah0

Abbreviations: mAb = Monoclonal antibody, CsA = microemulsion cyclosporine, Pred = prednisone, POD = postoperative day, borderline changes = suspicious for allograft rejection with no evidence of intimal arteritis but with foci of mild tubulitis (1 to 4 mononuclear cells / tubular cross section) and at least 10 to 25% of parenchyma inflamed, Type IA = cases with significant interstitial infiltration (> 25% of parenchyma affected) and foci of moderate tubulitis (> 4 mononuclear cells / tubular cross section or group of 10 tubular cells), Type IB = cases with significant interstitial infiltration (> 25% of parenchyma affected) and foci of severe tubulitis (> 10 mononuclear cells / tubular cross section or group of 10 tubular cells), Type IIA = cases with mild to moderate intimal arteritis, Type IIB = cases with severe intimal arteritis comprising > 25% of the luminal area, Type III = cases with transmural arteritis and / or arterial fibrinoid change and necrosis of medial smooth muscle cells.

Acute rejection score: t - 0 score = tubulitis; v - score = intimal arteritis; i- score = interstitial inflammation; g - score = early allograft glomerulitis; Chronic rejection score: ci - score = interstitial fibrosis; ct - score = tubular atrophy, cg - score = glomerulopathy; mm - score = mesangial matrix increases; cv - score = vascular fibrous intimal thickening; ah - score = arteriolar hyaline thickening.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject at least one antibody that recognizes a B7 antigen immediately prior to surgery and postoperatively administering to the subject at least one antibody that recognizes a B7 antigen immediately following surgery.

2. The method of claim 1, further comprising preoperatively administering at least one antibody that recognizes a B7 antigen at least about four days prior to surgery.

3. The method of claim 1, wherein two antibodies that recognize at least two B7 antigens are administered to the subject.

4. The method of claim 1, wherein at least one antibody is a humanized antibody.

5. The method of claim 1, wherein a higher dose of at least one antibody is administered prior to surgery than after surgery.

6. The method of claim 1, further comprising postoperatively administering at least one antibody that recognizes a B7 antigen at weekly intervals for at least about 3 months.

7. A method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject at least one antibody that recognizes a B7 antigen and postoperatively administering to the subject at least one antibody that recognizes a B7 antigen in combination with an immunosuppressive drug.

8. A method for downmodulating the immune response in a subject undergoing transplantation comprising preoperatively administering to the subject a combination of antibodies that recognize at least two B7 antigens and postoperatively administering to the subject a combination of antibodies that recognize at least two B7 antigens in combination with an immunosuppressive drug.

9. The method of claim 9, wherein the immunosuppressive drug is a rapamycin compound.

FIG. 1A

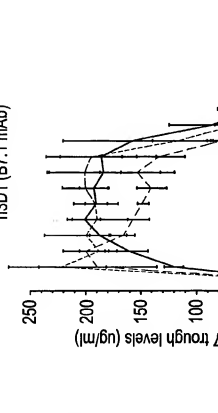

h3D1 (B7.1 mAb)
Anit-B7 trough levels (ug/ml)
250
200
150
100
50
0
h3D1 administration
0
21
42
70
112
Time post transplant (days)
Group I (mAbs mono)
Group II (mAbs + CsA)
Group II (mAbs + Pred)

FIG. 1B

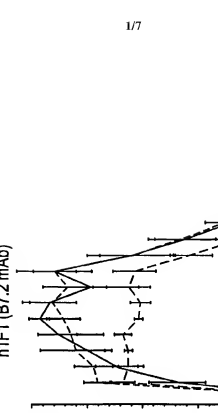

h1F1 (B7.2 mAb)
Anit-B7 trough levels (ug/ml)
250
200
150
100
50
0
h1F1 administration
0
21
42
70
112
Time post transplant (days)
Group I (mAbs mono)
Group II (mAbs + CsA)
Group II (mAbs + Pred)

FIG. 2

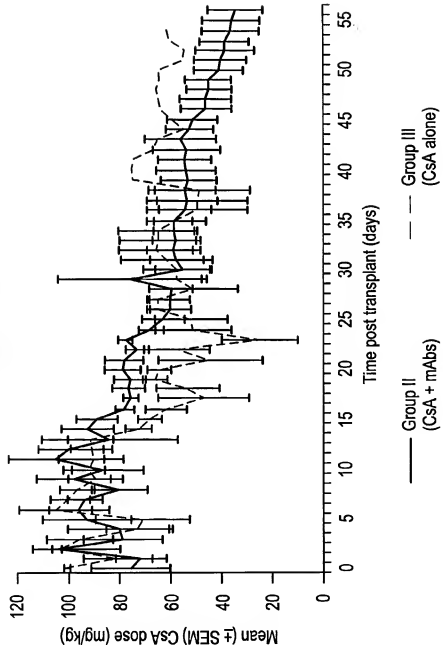

Mean CsA dose
Mean (± SEM) CsA dose (mg/kg)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
55
Time post transplant (days)
Group II
(CsA + mAbs)
Group III
(CsA alone)

FIG. 3

Mean CsA trough levels

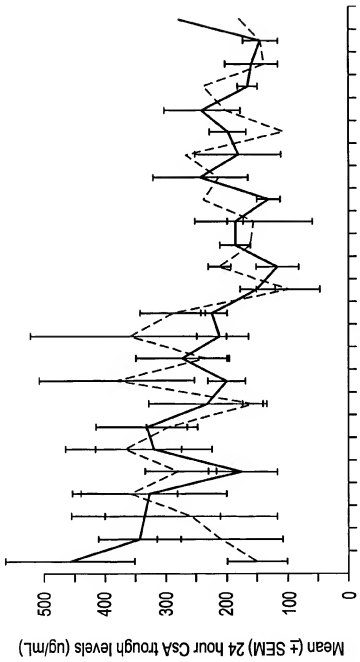


FIG. 4

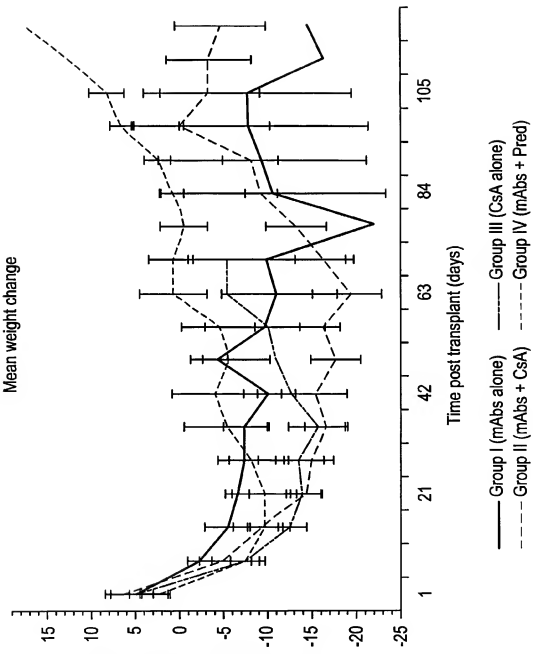

Mean weight change
Mean (± SEM) percent weight change
15
10
5
0
-5
-10
-15
-20
-25
1
21
42
63
84
105
Time post transplant (days)
Group I (mAbs alone)
Group II (mAbs + CsA)
Group III (CsA alone)
Group IV (mAbs + Pred)

FIG. 5A

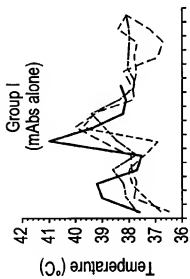
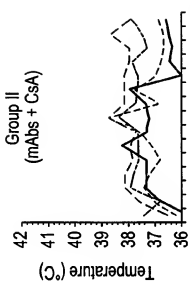

Group I
(mAbs alone)
Temperature (°C)
42
41
40
39
38
37
36
0
7
14
Time post transplant (days)
67485
98134
98133
98135

FIG. 5B


Group II
(mAbs + CsA)
Temperature (°C)
42
41
40
39
38
37
36
0
7
14
Time post transplant (days)
26117
118572
118104
126168

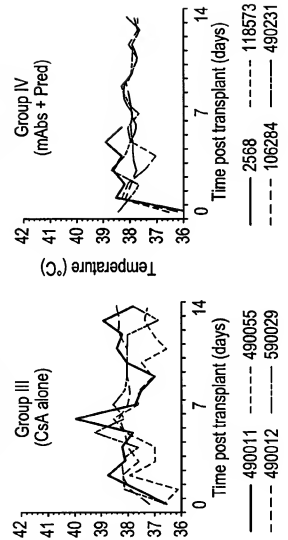
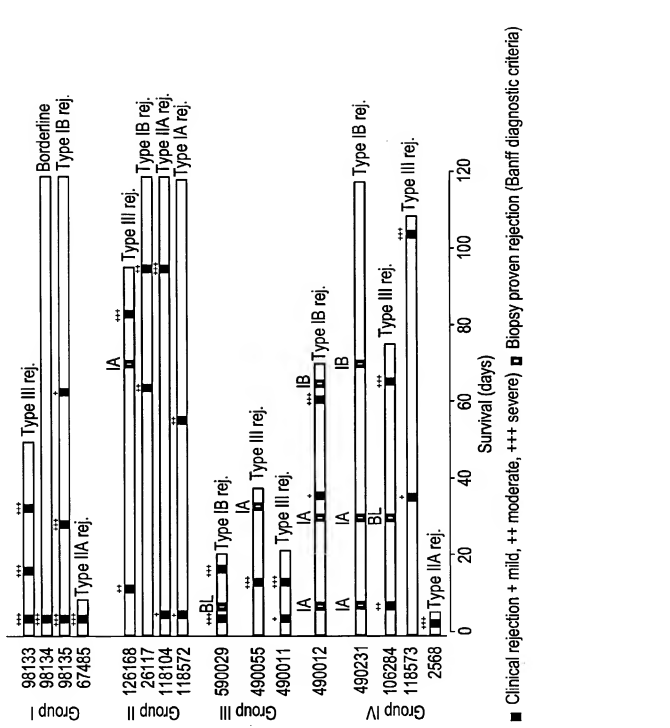


FIG. 5C
Group III
(CsA alone)
Temperature (°C)
42
41
40
39
38
37
36
0
7
14
Time post transplant (days)
490011
490012
490055
590029
FIG. 5D
Group IV
(mAbs + Pred)
Temperature (°C)
42
41
40
39
38
37
36
0
7
14
Time post transplant (days)
2568
106284
118573
490231

FIG. 6


Clinical and histopathological courses of individual monkeys
Group I
98133
98134
98135
67485
Type III rej.
Borderline
Type IB rej.
Type IIA rej.
Group II
126168
26117
118104
118572
IA
Type III rej.
Type IB rej.
Type IIA rej.
Type IA rej.
Group III
590029
BL
Type IB rej.
Group IV
490055
490011
490012
IA
IA
Type III rej.
IB
Type IB rej.
490231
106284
118573
2568
IA
BL
IB
Type III rej.
Type IB rej.
Type III rej.
Type IIA rej.
0
20
40
60
80
100
120
Survival (days)
Clinical rejection + mild, ++ moderate, +++ severe)
Biopsy proven rejection (Banff diagnostic criteria)

INTERNATIONAL SEARCH REPORT

In ernal Application No
PCT/US 01/08017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K31/445 A61P37/06 //(A61K39/395,31:445)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 58965 A (INNOGENETICS B.V.) 30 December 1998 (1998-12-30) page 99, line 24 -page 100, line 30 claims ---	1,3-5, 7-9
X	M. OSSEVOORT ET AL.: "Prevention of renal allograft rejection in primates by blocking the B7/CD28 pathway." TRANSPLANTATION, vol. 68, no. 7, 15 October 1999 (1999-10-15), pages 1010-1018, XP001016112 Baltimore, MD, USA cited in the application abstract	1,3,7,8
Y	--- -/--	4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 2001	07/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A. BREE ET AL.: "Humanized anti-B7.1 and anti-B7.2 antibodies prevent antigen-specific induction of immunity in nonhuman primates immunized with tetanus toxoid and mumps virus vaccine." BLOOD, vol. 94, no. 10, suppl. 1 (part 1 of 2), 15 November 1999 (1999-11-15), page 439a XP002175766 New York, NY, USA abstract# 1948 ---	4
X	B. BLAZAR ET AL.: "Infusion of anti-B7.1 (CD80) and anti-B7.2 (CD86) monoclonal antibodies inhibits murine graft-versus-host disease lethality in part via direct effects on CD4+ and CD8+ T cells." THE JOURNAL OF IMMUNOLOGY, vol. 157, no. 8, 15 October 1996 (1996-10-15), pages 3250-3259, XP002175767 Baltimore, MD, USA page 3251, right-hand column, line 40 - line 47 abstract ---	1,3,5
X	WO 95 34320 A (REGENTS OF THE UNIVERSITY OF MINNESOTA) 21 December 1995 (1995-12-21) examples 8,9 claims ---	1,3,5
A	A. KIRK ET AL.: "Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates." NATURE MEDICINE, vol. 5, no. 6, June 1999 (1999-06), pages 686-693, XP002175768 New York, NY, USA cited in the application abstract table page 687, left-hand column, line 6 -right-hand column, line 15 page 691, left-hand column, line 4 - line 21 --- -/--	1-9

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	T. PEARSON ET AL.: "Analysis of the B7 costimulatory pathway in allograft rejection." TRANSPLANTATION, vol. 63, no. 10, 27 May 1997 (1997-05-27), pages 1463-1469, XP001016121 Baltimore, MD, USA page 1464, right-hand column, line 2 - line 5 ---	1-6
P,X	WO 00 47625 A (GENETICS INSTITUTE, INC.) 17 August 2000 (2000-08-17) example 22 claims -----	1,3-9

INTERNATIONAL SEARCH REPORT

information on patent family members

Int ›nal Application No
PCT/US 01/08017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9858965 A	30-12-1998	AU 8800598 A	04-01-1999
		EP 0988321 A	29-03-2000
WO 9534320 A	21-12-1995	AU 2701895 A	05-01-1996
		CA 2191733 A	21-12-1995
		EP 0784482 A	23-07-1997
		JP 10501815 T	17-02-1998
WO 0047625 A	17-08-2000	AU 3998800 A	29-08-2000